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Valorization of food processing wastes into the fucose-rich polysaccharide FucoPol

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Resumo

A bactéria *Enterobacter* A47 é um produtor de exopolissacáridos que demonstrou ter a capacidade de utilizar diferentes fontes de carbono. Produz um exopolissacárido rico em fucose, denominado FucoPol que é composto por fucose, glucose, galactose e ácido glucurónico. O polímero possui propriedades interessantes ao nível reológico, floculante e emulsionante.

Neste trabalho, a polpa de maçã e o bagaço de uva, dois desperdícios gerados pela indústria das bebidas foram testados como substratos para o cultivo da *Enterobacter* A47. Ambos os substratos são ricos em açúcares, com um baixo teor em azoto, o que os torna adequados para a produção de FucoPol.

A fração solúvel da polpa de maçã, obtido pela centrifugação do resíduo, resultou numa produção de 6.10 g/L de EPS ao fim de 32 h no modo de cultivo fed-batch, o que corresponde a uma produtividade volumétrica de 5.63 g/L.d. O uso da fração solúvel filtrada em modo batch resultou numa produtividade volumétrica similar, mas o rendimento do polímero em açúcares simples foi melhorado 0.24 g/g.

O bagaço de uva foi processado em dois materiais ricos em açúcar usando água subcrítica (HCW) e hidrólise ácida diluída. O extrato de HCW mostrou-se não ser adequado para a produção de EPS, devido à sua baixa concentração em açúcares simples, mas o hidrolisado ácido resultou na produção de 3.92 g/L de EPS em 31 h de cultivo e num alto rendimento do polímero em açúcares base de 0.39 g/g.

O EPS produzido é composto por fucose (39-42 mol %), glucose (27-29 mol %), galactose (26-27 mol %) e ácido glucurónico (4-5 mol %), que é semelhante à composição típica do FucoPol, demonstrando desta forma que o polímero não foi significativamente afetado pelo uso dos diferentes substratos.

Palavras – chave: Exopolisacárido; FucoPol, *Enterobacter* A47; Desperdício resíduos alimentares; Água subcrítica; Hidrólise ácida

Abstract

The bacterium *Enterobacter* A47 is an exopolysaccharide (EPS) producer that has demonstrated the ability to use different carbon sources. It produces a fucose-rich exopolysaccharide, named FucoPol that is composed of fucose, glucose, galactose and glucuronic acid. This polymer has interesting properties at rheological, flocculation and emulsifying levels.

In this work, apple pomace and grape pomace, two wastes generated by the beverage industry were tested as substrates for the cultivation of *Enterobacter* A47. Both wastes are rich in sugars, with low nitrogen content, which makes them suitable for production of FucoPol.

The soluble fraction of apple pomace, obtained by centrifugation of the waste, resulted in the production of 6.10 g/L of EPS within 32 h of fed-batch cultivation, which corresponds to a volumetric productivity of 5.63 g/L.d. The use of filtered soluble fraction in batch mode resulted in a similar volumetric productivity but the polymer yield on a sugar basis was improved to 0.24 g/g.

Grape pomace was processed into two sugar-rich materials using hot compressed water (HCW) and dilute acid hydrolysis. The HCW extract was not suitable for EPS production due to its low simple sugars concentration, but the acid hydrolysate resulted in the production of 3.92 g/L of EPS within 31 h of cultivation and a high polymer yield on a sugar basis of 0.39 g/g.

The EPS produced was composed of fucose (39-42 mol %), glucose (27-29 mol %), galactose (26-27 mol %) and glucuronic acid (4-5 mol %), which is similar to the typical FucoPol composition, thus demonstrating that the polymer was not significantly affected by the use of the different substrates.

The results obtained demonstrated that apple pomace and grape pomace can be used for the production of FucoPol, reaching high volumetric productivities, without impacting on the polymer's composition. Since both substrates are low-cost, their use can contribute for the reduction of the production costs.

Keywords: Exopolysaccharide; FucoPol, *Enterobacter* A47; Food processing wastes; Subcritical water; Acid hydrolysis

Contents

List of Figures

List of Tables

Acronyms

DO – Dissolved oxygen concentration

CDW – Cell Dry Weight

C/N – ratio carbon/nitrogen

EPS – Exopolysaccharide

HCW – Hot compressed water

n.a. – Data not available

n.d. - Not determined

NMWCO – Nominal molecular weight cut-off

OD $_{450nm}$ – Optical density at 450 nm

Variable

r_p – Volumetric productivity (g/L.d)

$Y_{p/S}$ – Production Yield (g/g)

μ_{max} – Maximum specific growth rate (h^{-1})

Chapter 1

1. Introduction

1.1. Polysaccharides

Microorganisms can produce three types of polymers: intracellular, structural and extracellular polymers[1]. The word biopolymer or "renewable polymers" represents polymers that have been manufactured by biological systems, rather than chemically synthesized, using biological raw materials, such as amino acids, sugars, or natural fats[2]. Biopolymers can be used as an alternative to the chemical polymers because of their many advantages, like ease of biodegradability, high efficiency, non-toxicity and non-secondary pollution[3].

Polysaccharides are the biggest group of natural polymers that are produced in the world[4]. They have many applications in food, pharmaceutical, cosmetics, paints, explosives, paper and oil industries, and can also can be widely used as thickening, gelling, stabilizing, binding, emulsifying or flocculating agents since the variety of physical and structural properties is high [5]. Polysaccharides are produced by many living beings and it is possible to recover them from plants, algae, animal and microbial sources [4,6]. Normally, the use of microorganisms to produce polysaccharides is more appropriate than algae or plant, since microorganisms usually have higher growth rates and are more prepared for enhancing growth and/or production by changing the cultivation condicions[7]. In contrast with other sources (plant, algae, etc.), climate changes or seasonality do not influence microbial fermentation[5].

When the microorganism produces a polysaccharide and segregates it out of the cell, that polysaccharide is defined as an exopolysaccharide (EPS)[2]. Extracellular polysaccharides or exopolysaccharides (EPS) are expelled by the cell in two different ways: 1) as a capsule that remains attached to the cell surface, or 2) as a slime that is weakly connected to the cell surface[8]. Many bacterial have been reported as being capable of producing EPS, which are usually characterized by having high molecular weight and presenting an high diversity in terms of chemical structure, and composition[9].

EPS are produced by the cells under many different stress conditions and this gives to the cell some self-protection in case of predation, the effects of antibiotics, antimicrobial substances, antibodies and facilitates the adherence to bacterial and animal cells and plant tissue. EPS formation is affected by physical and chemical factors. The most important physical parameters are temperature, aeration, agitation and fermentation period, while the vital

chemical factors are media composition, source and concentration of carbon (C) and nitrogen (N), C/N ratio, trace elements, pH and dissolved oxygen (DO)[1,2].

EPS composition is significantly affected by microbial cells synthesis and, thereafter, a change in the chemical and physical properties[10]. EPS are principally composed of carbohydrates, but proteins and humic substances, even lipids, nucleic acids, uronic acids and some inorganic components, can also be present[11]. The most common sugar residues present in EPS structures are glucose and galactose[8].

Homo and heteropolysaccharides are the two groups that EPS can be divided into. Homopolysaccharide are polysaccharides formed by one kind of monosaccharide, normally D-glucose or L-fructose (e.g. dextran, levan)[3]. Heteropolysaccharides are composed usually of two to four different monosaccharides, which are arranged into a repeating unit constituted of up to ten monomers[12] (e.g. xanthan, gellan[3]). There some exceptions, such as alginate that has no defined repeating unit, with the number of monomers and their sequential distribution varies along the polymer chain and depends on the source of the alginate[13]. Many heteropolysaccharides also contain of acyl groups as additional adornments [14], examples of these are alginate, xanthan gum, gellan gum[3].

Bacteria are responsible for producing an extensive variety of EPS which are synthesized via different biosynthetic pathways[15]. Polymer synthesis involves a large number of reactions that can occur in various cell compartments, which makes this process a very complex step for the microorganism[16]. It starts with the entry of the substrate in the bacterium, which can enter in an active or passive way. After that, the substrate is catabolized by periplasmic oxidation or intracellular phosphorylation[17]. The periplasmic oxidative pathway exists only in certain bacteria, while the intracellular phosphorylative pathway is present in almost every bacterium. These systems have been described in numerous EPS-producing strains, and both these systems can work concurrently if there is enough substrate availability[18].

Some precursors perform as a raw material in EPS manufacture even if they do not participate in central metabolic pathways. For the polysaccharides synthesis, activated precursors are biosynthesized, that are monosaccharides with a high value of energy, mostly nucleoside diphosphate sugar (NDP-sugar), resulting from phosphorylated sugars. This vital phase is ruled by an independent pathway, where phosphorylated sugars, frequently appear under the form of sugar-1P and hardly under the form of sugar-2P or sugar-6P, is used as activated prime residue[17,18].

EPS are hydrophilic, high molecular weight polymers, which are assembled in the cytoplasm and have to cross the cell envelope without damaging the critical barrier properties. This is a complicated process for the bacterium[18]. For most gram-negative bacteria, the pathway for

biosynthesis and export of EPS can occur via one of two different pathways: the Wzx/Wzy-independent (ABC transporter-dependent) or the Wzx/Wzy-dependent pathway[2]. Wzy-dependent processes create a large variety of bacterial surface polymers. In such cases, a polymerase reaction is triggered in the periplasmic face of the plasma membrane. This, in turn, causes the need for the presence of an enzyme capable of transferring lipid-liked repeating units through the inner membrane[16]. The polymerization via ABC transporter-dependent happens at the cytoplasmic side of the inner membrane and the resulting polymer is then transferred through the inner membrane by an ABC transporter[2,16]. When the assemblage of repeating moieties at the lipid transporter, the backbone chain is translocated to the cell exterior, in the case of a gram-positive bacterium, where the elongation step will continue. However, instead of gram-negative is usually considered follow either Wzx-Wzy dependent or ABC transporter-dependent pathway[17].

In the case of Gram-positive bacteria, unlike gram-negative ones, the synthesis occurs in the exterior of the cell and is mediated by a range of extracellular enzymes[19].

1.2. FucoPol

FucoPol is a polymer composed of several sugars that are fucose (32 – 36 mol %), galactose (25 – 26 mol %), glucose (28 – 37 mol %) and glucuronic acid (9 – 10 mol %), and organic acids: succinyl (2 – 3 wt. %), pyruvyl (2 – 3 wt. %) and acetyl (3 – 5 wt. %)[20]. Fucose, galactose and glucose are neutral sugars, commonly found in EPS produced by members of the family Enterobacteriaceae[7,21]. FucoPol is a fucose-containing polysaccharide synthesized by the strain *Enterobacter* A47 (DSM 23139). It is a heteropolysaccharide with a high molecular weight (4.19×10^6 – 5.80×10^6), with different interesting rheological, flocculating, and emulsifying properties[9,22,23].

The presence of rare sugars, such as fucose, which are difficult to find in nature, gives this polysaccharide a higher market value with many potential applications, mainly in cosmetics and pharmaceuticals[24].

1.3. Polysaccharide Production

The biggest problem that limits the production of EPS at an industrial level, is the high costs of the most commonly used carbon sources (e.g. glucose, starch and sucrose)[5]. The costs of the substrate alone represent up to 40 % of the costs of all production of microbial polymers[9].

An alternative to those substrates is the use of considerably cheaper raw materials to reduce the overall costs of bioprocesses[24]. In EPS production, sucrose and glucose are normally the

substrates used, in contrast, xylose, galactose and lactose are less frequently used because several microorganisms are incapable of using them as a carbon source or the polymer synthesis is reduced. A few agricultural and industrial wastes, such as lignocellulosic material, cheese whey, and molasses, already have been proposed as carbon sources as an approach to reducing the polysaccharide production costs[24].

Enterobacter A47 has shown to be an extremely versatile bacterium due to its capacity to use a wide range of carbon sources[5], such as glycerol[25], glucose, xylose[22], and lactose, as well as several agro-industrial wastes or byproducts (glycerol byproduct from the biodiesel industry, cheese whey[26], tomato paste waste[27]). For this reason, the production of EPS by *Enterobacter* A47 may be economically viable for the use of other residues that are rich in sugars, such as residues from fruit processing or wine production.

1.4. Wastes Valorization

Production of waste matter is an irrefutable part of humanity. Wastes are produced by many sectors, including industries, forestry, agriculture, and municipalities. The "throw-away philosophy" and the growth of waste generation have severe consequences, such as, numerous ecological issues, health problems and safety hazards. This can be avoided by resource recovery and recycling of waste materials[28]. The industries of wine, milk, pulping and biodiesel represent a vital part of the Portuguese economy and they are responsible for the production of high quantities of industrial residues[29]. The treatment, discard and administration of these wastes is a big challenge for industries, urban local bodies, scientists and engineers. In recent years, the rising concerns about the environment has led authorities to search for new economically viable solutions for recycling and/or valorizing waste products[30].

1.5. Apple Pomace

Large amounts of liquid and semi-solid wastes are produced from food processing industries, livestock and poultry farm[31]. One of the major industries in the world is the fruit juice and is responsible for producing huge amounts of wastes, that include peels, seeds, pomace, rags, kernels, etc[32]. The production of apple was more than 70 million tons in 2015, of which the European Union contributed with more than 15 %[33]. Only 40 to 60%, depending on the year, of all the apple production is worn by the industry[34]. The drinking manufacture used about 15 % of this production, especially for juice and cider[35]. During the process of producing beverages, high amounts of solid residues are build-up, compound principally by peels, seeds, and pulp, which

are jointly known as apple pomace[36]. Even though apple waste is not polluting as another vegetable by-products coming from the food industry, it is a worthless material that is often discarded without control or sent to landfills for treatment, with associated costs[37].

Disposable material from some industries can be hold as renewable raw materials for production of other resources and commodities[33]. Some industries that process apples already showed some interest in the search for practicable alternatives both economically and technologically[38]. According to some studies, apple pomace could be used in the production of some added-value products (bioactive molecules – natural antioxidant[39]; fiber sources – pectin[40]; ursolic acid[41]), as a substrate or media for microbial growth. Basically, all the compounds that make up the apple like polymers, such as cellulose, hemicellulose, pectin and lignin, simple sugars like glucose, fructose and sucrose, even some few amounts of acids, minerals, proteins, vitamins and others, stay in the pomace[33]. Apple pomace can be used as a substrate because it is rich in sugars that can be used by many microorganisms. Therefore, apple pomace valorization is important to reduce its wastage, as well as its environmental impact by avoiding to be dumped into the environment[42], their use in the production of biotechnological products can also contribute to reduce the production costs of these processes.

1.6. Grape Pomace

One of the most important agricultural activities in the world is wine production[30]. Nowadays, there is a rising interest in the exploitation of the remains produced by the wine industry[43]. Grapes have an annual production of over than 67 million tons, which makes this fruit the largest harvest one in the world. It is cultivated principally for vinification as *Vitis vinifera*, and about 80 % of all grapes in the world is used to make wine[44,45]. The juice is formed by pressing the grapes and, from that method results a residue called grape pomace or marc. This residue represents 16 % of the original fruit and is formed from the skins, seeds and, pieces of stem[46]. To produce red wine, the seeds and skins are frequently in contact with the yeast broth for several days[45].

Winemaking is a seasonal activity that occurs normally just during autumn. After 3 months of the starting process, 60 to 70 % of the liquid streams are generated[30]. Afterward, the fermentation step, produces millions of tons of grape pomace, which is a challenge for the waste management issue both environmental and economically[45]. Vinification produces diverse wastes characterized by high contents of biodegradable compounds and suspended solids, containing a significant amount of lipids, proteins, non-digestible fibers and minerals[30,47].

In the winery waste, the lignocellulosic material is the amplest and useless residue[30]. Annually, the production of lignocellulosic waste generates a pollution problem to the environment in all the world, which represents a wastage of rich material that can be bioconverted into several added-value products (biogas and bioethanol[28])[48]. The interest in lignocellulosic biomass processing as grown, mainly on agricultural and forestry residue, once they are cheaper, abundant, promptly available and renewable[49].

Lignocellulose is a complex material that has a miscellaneous composition and can be reluctant in the transforming reactions. It is composed principally of cellulose (40–50 %), hemicellulose (25–30 %) and lignin (15–20 %), being responsible for the structural backbone of every plant cell walls[50]. Hemicellulose is composed of macromolecules, normally polymers of pentoses (e.g. xylose and arabinose), hexoses (e.g. mannose), uronic acids (e.g. galacturonic acid, glucuronic acid, 4-*O*-methyl-glucuronic[51]) and some sugar acids (e.g. tartaric acid[52]), whereas cellulose is a homogenous polymer composed solely of glucose. Lignin is composed of an aromatic polymer and it is synthesized from phenylpropanoid[53,48].

Almost all the cellulose, hemicellulose and lignin that are formed as by-products of agriculture or forestry are considered as wastes, just a little bit is used[48]. Some bioprocesses using grape pomace as a raw material to produce fine products have been proposed[54]. This is the case of fermentative utilization, the grape pomace residue is used as bacterial substrate for the development of diversity products with value-added products[39] (microbial polysaccharide – pullulan/xanthan production[43], alcohols - ethanol, single cell protein, methane, fine chemicals[48]).

1.7. Lignocellulosic Fractionation

The lignocellulosic material is mainly composed of cellulose (40-50 %), hemicellulose (25-30 %) and lignin (15-20 %). Its complex chemical composition and compact multilevel structure make the lignocellulosic material difficult to biodegrade. Hemicellulose is a polysaccharide composed of various sugar monomers, such as xylose, galactose, mannose, arabinose, and glucose, that render it a non-crystalline structure. Cellulose, on the contrary, is a homopolymer consisting of D-glucose monomers β-1-4 glycosidic linkages that are further stabilized by intrachain hydrogen bonding. This gives cellulose a linear structure. Lignin has a random three-dimensional structure, consisting of three monomers that are conifery alcohol, sinapyl alcohol and p-coumaryl alcohol. This structure provides strength and resistance to enzymatic degradation. Of the three components, lignin is the most recalcitrant to degradation[50,55,56,].

Hence, prior to their use as substrate for microbial cultivation, lignocellulose materials usually require a pretreatment that modifies their physical and chemical properties, transforming them into fermentable sugars[57,58]. Pretreatment has been considered one of the most expensive process steps for converting the cellulosic biomass to fermentable sugars[59].

In order to fractionate the lignocellulosic materials and obtain high sugar yields from both cellulose and hemicellulose, several pretreatment methods are available[60,61]. The pretreatment can be categorized as biological (microbiological and microaerobic treatment), chemical (acid and alkali hydrolysis), physical (mechanical, steam explosion, microwave radiation), or thermal process[62]. The physical and biological pretreatments have the advantage of being simple and have low consumed of chemical and energy, but the yields in sugars are too low or the time spent in obtaining them is too high. Chemical and thermal processes have the advantages of recovering high sugar yields and are faster processes, but more expensive and/or polluting[60].

1.8. Acid Hydrolysis

Sulfuric acid (H_2SO_4) is the typical acid used, however, hydrochloric acid (HCl), nitric acid (HNO_3), and phosphoric acid (H_3PO_4) can also be applied[63]. For a better hydrolysis, there are some parameters to be taken into consideration, such as acid concentration, reaction temperature and time, these are the main process parameters that can affect the efficiency of the treatment[61]. In the case of acid hydrolysis, it can be basically separated into two methods, based on concentrated acid and low temperature (<100 °C), or dilute acid and high temperature (200–240 °C[64])[63].

Concentrated acids are very effective agents for lignocellulosic materials hydrolysis but, it is an extremely expensive method. Furthermore, it has many associated problems, such as a need for corrosion resistant reactors, operational problems and also the fact of being toxic and hazardous to the environment[58,63]. To make a more sustainable method from an economical point of view, the concentrated acid should be recovered after hydrolysis[58].

Pretreatment with dilute acid has received more attention as it is a relatively cheaper process than pretreatment with concentrated acid, as well as, efficient in treating different biomass species. In addition, the dilute acid method is capable of, solubilizing hemicellulose and converting it into fermentable sugars, including monomeric sugars (xylose, arabinose, galactose, glucose, and mannose) and oligomers. This process is a more favorable process for industrial application because even at moderate temperatures (100 to 150 °C[65]), the pretreatment with dilute acid is capable of, rendering the hemicellulose, from lignocellulosic materials, practically complete, thus having a relatively high sugar yield. Due to, the emergence of new

microorganisms with the ability to use sugar consisting of pentoses and hexoses, as an energy source, the pretreatment of dilute acid becomes a more viable step in the hydrolysis of lignocellulosic materials[61]. In addition, dilute acid pretreatment methods compared to concentrated acid hydrolysis, create much less degradation products, as well as, fewer corrosion problems in hydrolysis tanks, pipes, etc[63].

The main disadvantage in the use of acid hydrolysis in the preparation of hydrolysates is the formation of byproducts, which inhibit or prevent their fermentation, therefore, the higher the concentration of the acid and/or the reaction temperature, the greater the number of such degradation products in the hydrolyzed[61,59]. To avoid the formation of these inhibitors, the reaction conditions must be taken into account, to maintain the formation of these byproducts at low levels, since the type of inhibitors formed and their concentration depend on the degree of harshness of the hydrolysis reactions[66]. The degradation products include acetic acid, formic and levulinic acids, phenolic, furfural and 5-hydroxymethylfurfural compounds, which are formed by the degradation of various products, for example, from the degradation of the hemicellulose structure or produced by the degradation of the sugar (pentose and hexose)[61].

1.9. Subcritical Water Extraction

Among the different pretreatment processes for sugar extraction, hydrothermal processes, where water is the only catalyst, have been widely used to solubilize sugars from softwood, hardwoods, energy crops and agricultural residues. A major advantage of the hydrothermal pretreatment process is the fact that it does not use any external chemicals, although it is a relatively more expensive method comparing to dilute acid hydrolysis[59]. The use of pressurized hot water to for extract and hydrolyzer is a very promising energy-efficient and environmental friendly technique[67]. Using water as an extraction solvent is very important from the ecological point of view because water is considered the greenest solvent that can be used in an extraction process[68]. The properties of water as a solvent are nonflammable, nontoxic and easy to obtain, as well as, safe, cheap and more environmental benign that organic solvents[69].

The term “pressurized hot water” is used to designate the condensed phase area between the boiling point (100 °C) to the critical point of water (374 °C) where the pressure is applied so that water remains in its liquid state[70,71]. Further terms have been also used, like “subcritical water”, “superheated water”, “near critical water”[70]. Henceforth, only the terms subcritical water and hot compressed water (HCW) will be used. Subcritical water cannot be defined as a physical state because all water beneath the critical point and above the triple point only can be liquid or gaseous[72].

The water physical and chemical properties can change drastically if the conditions of pressure and temperature also change, with the rising of the temperature, the dielectric constant (Ɛ) decreases abruptly from 80 at 25 °C to 20 – 30 at 200 – 350 °C, due to the dissociation of the hydrogen bonds. This causes the solubility of the hydrophobic molecules to increase, while the solubility of the ionic molecules decreases, thus enabling the extraction of compounds[71,73]. Another property of the water that changes with the increasing of the temperature is the ionic strength, the ionic product of the water (Kw), can changes from $Kw=10^{-14}$ at 25 °C to $kw=10^{-11}$ at 300 °C, this rising in the Kw increases the H^+ and OH^- concentration, making water a more reactive medium, capable of behaving as reagent as well as solvent [74,75].

The hydrothermal processes used to remove the hemicelluloses can be observed due to the already known ability of the water to hydrolyze polysaccharides. Although it is a very simple concept, in fact this type of hydrolysis is a very difficult process, because the hemicellulose-lignin-cellulose structure is very resistant and complex[76,72]. This process has many advantages over acid and enzymatic hydrolysis, since it is a clean and fast method, does not need to use toxic solvents, has a shorter reaction time, less corrosion, generates less waste, and induces a lower formation of degradation products[72]. Besides from the use for hydrolyzing lignocellulosic material, the hydrothermal process can be used for extracting some composts of that material or just fractions of the biomass.

1.10. Motivation

The high demand of society for industrial products led to an increase in the production of wastes, reducing the availability of raw materials and increasing their cost[77].

The food industry is responsible for producing large quantities of waste, which in addition to being a great loss of valuable materials, generates enormous problems of environmental and economic management. Many of these wastes produced by the food industry contain numerous substances of high commercial value that can be reused or valorized into value-added products[77,78]. Normally the residual organic composition present in this type of waste includes about 75% sugars and hemicellulose, 9% cellulase and 5% lignin[78].

For example, the beverage industry annually produces about 5 - 9 million tonnes of grape pomace and 3 - 4 million tonnes of apple pomace[79]. These wastes can be used as substrate for the growth of microorganisms and the production of value-added products, as is the case of exopolysaccharides that have a wide range of applications due to their functional properties.

Using this type of substrates can make the production processes cheaper and, at the same time, helps in the management/disposal of wastes[12,39].

Chapter 2

2. Materials and Methods

2.1. By-products characterization

2.1.1. Characterization of grape and apple pomace

2.1.1.1. Density

The density of the apple pomace was determined by weighing an empty graduated cylinder, then placing 20 mL of apple pomace and weighing again. Five replicas were made.

2.1.1.2. Viscosity

The viscosity of the apple pomace was determined with a viscometer (Fungi Lab S.A., Alpha series, Spain). As the apple pomace is very viscous, it was necessary to dilute it (1:2, 1:3 and 1:4) in order to be able to measure the viscosity, as well as to choose the best dilution to be used in the bioreator.

2.1.1.3. Total dry mass

A centrifuge tube (10 mL) was filled up to half with apple pomace, weighed and lyophilized.

2.1.1.4. pH and Conductivity

For the grape and apple pomace was recorded its pH (pH1100L, VWR pHenomeral™) and its conductivity (Five easy conductivity, Mettler Toledo).

2.1.1.5. Total nitrogen

For total nitrogen determination, a kit (LCK 388, LATON®) with a detection range of 20-100 mg/L was used. The test solution (0.2 mL) was placed into a digestion flask; then, the reagents were added as described in the kit and the flasks were placed on the HT 200S (HACH®-LANGE) digester for 15 min at 100 °C. The flasks were cooled to room temperature. After cooling, the flasks were agitated and 0.5 mL of the solution was transferred to a new flask and after 15 min the absorbance was read on a DR 2800 tm spectrophotometer (HACH®).

2.1.1.6. Ammonia and Phosphorus

The ammonium and phosphates content was determinate using a Skalar equipment (Skalar 5100, Skalar Analytical, The Netherlands) and the samples were diluted to within the range of 4 – 20 ppm. The standard solutions were prepared using phosphorus (KH_2PO_4) and ammonia (NH_4Cl) in milli-Q water in concentration of 4 – 20 ppm.

2.1.1.7. Suspended and Dissolved material

The dissolved and suspended material was determined by placing 6 mL of grape or apple pomace. After centrifugation at 7012x *g* for 15 minutes (in the case of apple pomace, the samples were diluted), the supernatant was lyophilized (Scanvac, CoolSafe).

2.1.1.8. Salt content

In order to determine the salt content died pomaces samples, supernatants, and pellets, (about 0.1 g) were placed in a muffle at 550 °C for 2h, after cooling to room temperature, the samples were weighed to quantify their inorganic materials contents.

2.1.1.9. Soluble polysaccharide content

For determination of the soluble polysaccharide content, samples of grape and apple pomace (diluted 1:4) were centrifuged for 15 min at 7012x *g*. The resulting supernatant (3 mL) was dialyzed with with a 12000 MWCO membrane (ZelluTrans Carl Roth Cellulose Membrane SO farblos) against deionized water. The dialysis was performed in a 5 L bucket with a constant stirring and added 10 ppm of sodium azide to avoid cellular growth. The water was changed 3 to 4 times a day until the conductivity value was below 10 µS/m (around 72 h). After dialyzed the samples were lyophilized (Scanvac, CoolSafe) during 48h and weighed.

2.1.1.10. Determination and Quantification of sugar

To determine the sugars present in the grape and apple pomace, the samples were prepared in two different ways to be analyzed by HPLC. The first was just analyzed the supernatant, the second way the supernatant and the pellet were hydrolyzed in 100 µL of TFA at 120 °C for 2h after that, were analyzed in the Dionex 3000 chromatograph with an AminoTrap column (BioLC Termo Dionex) and a CarboPac PA10 250x4 mm column (Thermo Dionex). The standards D-(+)-

glucose (99 %, Fluka), D-(+)-galactose (99 %, Fluka), D-(+)-mannose (99 % Fluka), L-rhamnose monohydrate (99 %, Fluka), D-glucuronic acid (98 %, Alfa Aesan), xylitol (99%, Sigma), D-(+)-Trehalose dihydrate (99 %, Alfa Aesan), D-arabinose (99 %, Sigma), sucrose (99 %, Fluka), D-(+)-galacturonic acid (97 %, Fluka) and D-(+)-xylose (9 %, Merck) were prepared in deionized water with a concentration of 1 g/L.

2.1.1.11. Granulometry

The particle size distribution of the grape pomace was determined using sieves (Laboratory Test Sieve, Endecotts LTD., London England) with pores sizes between 1400 µm and 125 µm. Each weight fraction was weighed separately.

2.1.2. Appel pomace characterization

The apple pomace was supplied by Sumol + Compal, S.A., several preliminary tests were done to determine the characteristics of apple pomace, such as viscosity measurement at ratios of 1: 2,1: 3 and 1: 4, pH measurement and conductivity. Its density, total nitrogen, total dry mass, dissolved and suspended material and the content of polysaccharides and salts were determined. Its composition in sugars was also determined and the total sugars were quantified.

2.1.2.1. Apple pomace medium

In order to be able to use the apple pomace as a medium, had to be diluted in the ratio of 1:3, because the apple pomace was too viscous and it was intended to separate most of the insoluble solids from it. This solution was centrifuged at 7012x *g* for 30 min for its sterilization. After that, the solution was autoclaved at 121 °C for 30 min. The feed solution for the fed-batch bioreactor experiments was prepared the same way. The solutions were supplemented with K_2HPO_4, KH_2PO_4, $((NH_4)_2HPO_4)$, $MgSO_4$ and micronutrients, in the appropriate amounts to give the same concentration of Medium E* (composition described below).

2.1.2.2. Apple pomace filtered medium

For preparation of the filtered medium, apple pomace was diluted and centrifuged as described above, and then filtered using a cross-flow module (Sartocon Slide Holder), equipped with a 100 000 Da nominal molecular weight cut-off (NMWCO) ultrafiltration membrane (Hydrosart® Ultrafiltration Cassette, Sartorius), with a surface area of 100 cm^2, operated at a

transmembrane pressure below 1.5 bar. After filtration, the medium and feed solution was autoclaved at 121 °C for 30 min. The solution was supplemented with K_2HPO_4, KH_2PO_4, ($(NH_4)_2HPO_4$), $MgSO_4$ and micronutrients, in the appropriate amounts to give the same concentration of Medium E*. The feed solution for the fed-batch bioreator experiments was prepared the same way.

2.1.3. Grape pomace characterization

The red grape pomace was provided by a Portuguese wine producer, from Alentejo region, and were part of the last wine production (2016). It was stored at -20 °C until use. The grape pomace was dried in an oven at 80 °C overnight; then, the dry grape pomace was ground into a powder (grape pomace powder) and stored in a closed bag in the freezer at -20 °C. The powder granulometry was determined, as well as the pH, conductivity and total nitrogen of the grape aqueous extract. Their content in dissolved and suspended matter and salts, as well as the composition in sugars and their content in total sugars, were also evaluated.

2.1.3.1. Grape pomace medium

2.1.3.2. Acid Hydrolysis

To obtain simples sugars, it was necessary to hydrolyze the grape pomace powder. The hydrolysis was performed in two steps. The first step the extraction of soluble material from the grape pomace, which was performed by mixing 461 g of grape pomace power in 2 L deionized water and autoclaving at 121 °C for 30 min. After cooling to room temperature, the mixture was centrifuged at 7012x *g* for 30 min to separate the solids fraction. The supernatant was collected and filtered (47 mm, glass microfibres filter, 691 - VWR), to remove some solids which were still in suspension and which could interfere in the production process. The second step was to hydrolyze the filtered solution using sulfuric acid (H_2SO_4), was added until the pH≈2 with a final H_2SO_4 concentration of ≈3 % (21 mL of H_2SO_4, 95-97 % Sigma aldrich). Then, the solution was autoclaved at 121 °C for 30 min, after cooling to room temperature, the solution was neutralized with ≈ 50 g/L of NaOH pellets.

2.1.3.3. Subcritical Water Extraction

In the extraction with subcritical water (Fig. 1), the reactor is placed in an electric oven with temperature control. The reactor used was a 51 cm long stainless-steel tubular reactor, 5 cm

outside diameter, 2.6 cm inner diameter (TOC7-20-G REACTOR, HiP High Pressure Equipment Company, USA). The system pumps distilled water through a high-pressure pipe, the water passes through a filter and then is heated by a heating wire before reaching the reactor, a thermocouple monitors the water temperature before entering the reactor. The water leaving the reactor goes through a filter again and its temperature is controlled again. The pressure of the system is controlled at the outlet of the reactor by a back-pressure regulator (BPR; Tescom Europe®, 26-1000). The valves and accessories used are HIP and SWAGELOK.

Before placing the reactor in the oven, a porous disk was placed in the exit of the reactor and this is filled with the grape pomace powder (≈50 g) mixed with small glass beads (≈300 g). To begin the experiment, the pump was switched on at the selected flow rate (10 mL/min) and the BPR was set to the desired pressure. When the pressure reached 80 bar, the water heating wire and the oven were turned on (200 °C), and starting to collect the grape pomace extract. The water temperature was increased slowly (about 120 min) until the outlet temperature reach the desired final temperature (200 °C). Then, the temperature was kept constant for 30 minutes. The grape pomace extract was stored at -4 °C until use in the bioreactor experiments.

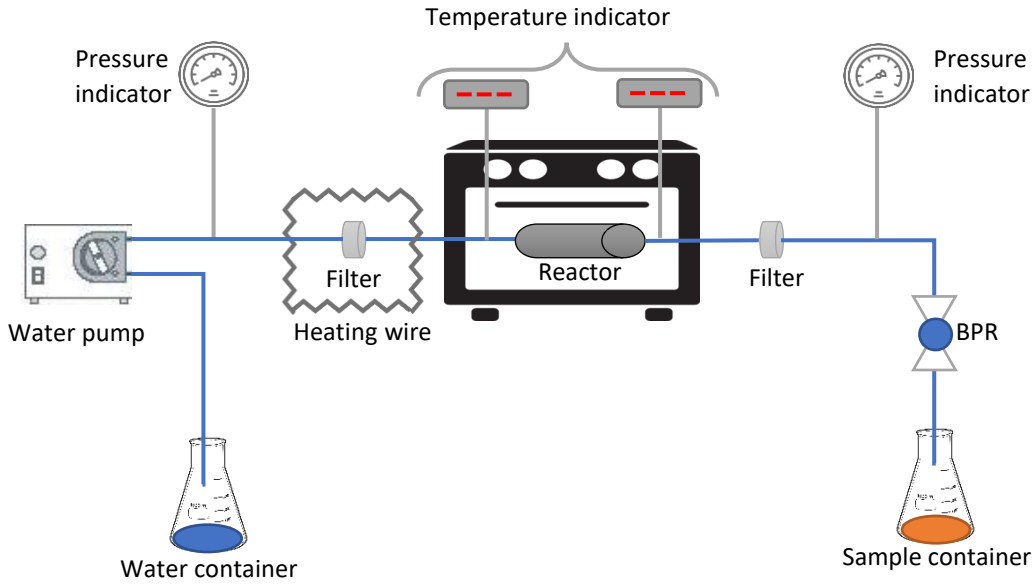

Figure 1: Schematic of the subcritical water experimental set-up.

2.2. EPS production

2.2.1. Microorganism

The microorganism used for the FucoPol production was the *Enterobacter A47* (DSM23139). The microorganism was preserved in 20 % (v/v) glycerol and stored at -80 °C.

2.2.2. Media

2.2.2.1. Pre-inocula

Luria Broth - Composition (per litre): tryptone, 10.0 g; yeast extract, 5.0 g; and NaCl, 10.0 g. To performed the pre-inocula was used a 50 mL erlenmeyer with 20 mL of LB at pH 7.

2.2.2.2. Inocula

Medium E* (Slightly modified) - Composition (per litre): $(NH_4)_2HPO_4$, 3.3 g; K_2HPO_4, 5.8 g; HK_2O_4P, 3.7 g. 10 mL of a 100 mM $MgSO_4$ solution; and 1 mL of micronutrient solution. This solution was composed (per litre of 1 N HCl): $FeSO4.7H_2O$, 27.8 g; $MnCl_2.4H_2O$, 1.98 g; $CoSO_4.7H_2O$, 2.8 g; $CaCl_2.2H_2O$, 1.67 g; $CuCl_2.2H_2O$, 0.17 g; and $ZnSO_4.7H_2O$, 0.29 g. To the medium was added yet ≈ 40 g/L of glycerol, for the inocula, or one of the waste substrates, as described below.

2.2.3. Cultivation conditions

To reactivate the cryopreserved stock culture, it plated on CHROMagar™ Orientation, and incubated during 48 h at 30 °C. Thereafter, the pre-inocula was prepared by inoculating an isolated colony into 20 mL of LB medium, in a 50 mL shake flask, and incubation during 24h at 30 °C and 200 rpm, in an orbital shaker (New Brunswick Scientific). After that, 10 mL of pre-inocula was transferred into 100 mL of Medium E* (in a 500 mL shake flask) and further incubated for 72 h in the same conditions to obtain the incula for the bioreator experiments. To prevent contamination and ensure the maintenance of sterile conditions, all the manipulations were performed in a laminar flow chamber and the solutions used were sterilized previously in the autoclave (20 min, 121 °C, 1 bar).

2.2.4. Bioreator operation

For the experiments, a 2 L bioreator (Biostat B, Sartorius, Germany) was used. The system provides an automatic control of temperature, pH, mechanical, stirring, foam control and dissolved oxygen concentration[22].

2.2.5. Apple pomace assay

2.2.5.1. Batch operation

The batch assays were performed in ≈ 30h with a starting volume of 1.5 L of apple medium, with an initial sugars concentration of ≈ 40 g/L and supplement with Medium E* with the following composition: 6.96 g K_2HPO_4, 4.44 g KH_2PO_4, 3.96 g ($(NH_4)_2HPO_4$) diluted in 40 mL of deionized water, and 12 mL of a 100 mM $MgSO_4$ solution and, 12 mL of a micronutrient solution diluted 1:10.

2.2.5.2. Fed-batch operation

The fed-batch assays were performed in a total time between 45 to 59h, with an initial volume of 700 mL of medium with an initial concentration of sugars between 36 and 49 g/L, the medium was supplement with: 3.48 g K_2HPO_4, 2.22 g KH_2PO_4 and, 1.98 ($(NH_4)_2HPO_4$) diluted in 20 mL of deionized water, and 6 mL of a 100 mM $MgSO_4$ solution and, 6 mL of micronutrient solution diluted 1:10. After an initial batch phase of 8 h, the bioreactor was fed with substrate with a constant feed-rate of 30 mL/h, the fed was equal to the initial medium and supply with medium E*, 1.16 g K_2HPO_4, 0.74 g KH_2PO_4 and 0.66 g ($(NH_4)_2HPO_4$) diluted in 10 mL of deionized water and 2 mL of a 100 mM $MgSO_4$ solution and 2 mL of micronutrient solution 1:10, the initial feed volume was 1.8 L.

2.2.6. Apple pomace filtered assay

In this experiment, the batch and fed-batch assays were performed as described above, only changing the medium. The batch assay took 28 h and the fed-batch took 59 h and both with an initial sugar concentration of ≈ 30 g/L.

2.2.7. Grape pomace assay

The grape pomace assays were only performed in fed-batch as described above for the apple pomace, either for the acid hydrolysis or subcritical water. The assay using the medium obtain from acid hydrolysis, was performed in 45 h with an initial sugar concentration of ≈ 4 g/L. The medium obtain from subcritical water extraction was performed in 48 h with an initial sugar concentration of ≈ 0.05 g/L.

In all assays, the temperature was controlled at 30 ± 0.2 °C, the pH was established at 7 ± 0.05 by using NaOH (2M) and HCl (2M) solutions previously prepared and the aeration rate was settled at 0.4 standard litre per minute (SLPM) and sustained through all cultivations. Through the automatic variation of the stirrer speed (300-800 rpm) the dissolved oxygen level (DO) was maintained at 10 %.

Throughout the assay samples (24 mL) were collected every 2 or 3 h in order to monitor cell growth. The biomass, polysaccharides, and nutrients were quantified in the samples collected from the bioreactor and the apparent viscosity was recorded.

To obtain a cell-free supernatant, the broth samples were centrifuged at 7012x *g* for 15 min (Sigma 4-16 Ks, Germany) to separate the biomass from the cell free supernatant, in the case of more viscous samples, they were diluted (1:2, 1:3, 1:5 or 1:6) with deionized water to reduce viscosity. The samples were stored at -20 °C for further analysis of the concentration of phosphorus and ammonia, for quantification of EPS and determination of sugar concentration.

2.3. Analytical techniques

2.3.1. Cell growth

The cell growth was evaluated by measuring the optical density at 450 nm (OD_{450nm}) (VWR V-1200 spectrophotometer, Portugal) of the broth samples withdrawn from the reactor. Three replicas of each sample were made to give greater certainty to the result.

The cell dry weight (CDW) was estimated considering that one unit of OD_{450nm} is equivalent to a CDW of 0.26 g/L[27].

$$CDW = 0.26\ x\ OD450 \qquad \text{(Equation 1)}$$

2.3.2. Apparent viscosity

To evaluate the evolution of EPS production, the viscosity of the samples was measured with a viscometer (Fungi Lab S.A., Alpha series, Spain), the rotational speed ranging from 100 – 0.3 rpm. Samples out of range were not measured.

2.3.3. Sugar concentration

Sugars concentration in the cell free supernatant was determined by high-performance liquid chromatography (HPLC) using Dionex 3000 chromatograph with an AminoTrap column (BioLC Termo Dionex) and a CarboPac PA10 250x4 mm column (Thermo Dionex). The analysis

was performed at 30 °C, with sodium hydroxide (18 mM NaOH) as eluent with an injection of 10 µL, at a flow rate of 1 mL/min. The samples were prepared in deionized water and diluted so that the concentration was below 100 ppm. Standards were prepared using D-(+)-glucose (99 %, Fluka), D-(+)-galactose (99 %, Fluka), D-(+)-mannose (99 % Fluka), L-rhamnose monohydrate (99 %, Fluka), D-(+)-Trehalose dihydrate (99 %, Alfa Aesan), D-arabinose (99 %, Sigma), and D-(+)-galacturonic acid (97 %, Fluka) with a concentration between 0.1 g/L and 0.001 g/L.

2.3.4. Exopolysaccharide quantification

EPS production across cultivation was evaluated by extraction of the polymer from the cell-free supernatant by dialysis. The cell-free supernatant samples (3 mL) were dialyzed with a 12000 MWCO membrane (ZelluTrans Carl Roth Cellulose Membrane SO farblos) against deionized water. The dialysis was performed in a 5 L bucket with a constant stirring. Sodium azide (10 mL) added to avoid sample microbial degradation. The water was changed 3 to 4 times a day until the conductivity value was below 10 µS/m (around 72 h). Hereafter, the dialyzed samples were freeze dried (Scanvac, CoolSafe) during 48h. Then, the samples were weighed to determine EPS production and kept for further characterization.

2.3.5. Exopolysaccharide composition

EPS dried samples (≈ 5 mg) were dissolved in deionized water (5 mL) and hydrolyzed with trifluoroacetic acid (TFA) (0.1 mL TFA 99 %) in a dry bath at 120 °C during 2h. The hydrolysate was used for the identification and quantification of the constituent monosaccharides by Dionex 3000 chromatograph with an AminoTrap column (BioLC Termo Dionex) and a CarboPac PA10 250x4mm column (Thermo Dionex) equipped with a PAD detector.

The analysis was performed at 25 °C, with sodium hydroxide and sodium acetate as eluent, at a flow rate of 1 mL/min. The standards D-(-)-Fucose (98 %, Scharlau), D-(+) glucose (99 %, Fluka), D-(+)-Galactose (99 %, Fluka), D-(+)-mannose (99 % Fluka), L-rhamnose monohydrate (99 %, Fluka) and, D-glucuronic acid (98 %, Alfa Aesan) were prepared in deionized water in concentrations between 5 ppm and 100 ppm.

2.3.6. Total sugars

This method uses a calibration curve to quantify the total sugar content of sugar-rich liquors. The calibration curve was constructed with solutions of D-(+)-glucose monohydrate (Sigma Aldrich) at concentrations of 0.1, 0.05, 0.025, 0.01 g/L. The blank was deionized water.

To carry out the analysis, for 500 µL of standard or liquor samples, added 1.5 mL of H_2SO_4 (Panreac 96%) and 300 µL of a 5 % (w/v) aqueous solution of phenol (Sigma Aldrich 99-100%). Soon after, the mixtures were well stirred and incubated for 5 min at 90 °C in a dry bath (Accu BlockTM Digital Dry Bath). Thereafter, the mixtures were stirred again and, cooled in a water bath for the room temperature. Absorbance is measured at 490 nm (DU®800 Spectrophotometer from Beckman Coulter, Brea, USA). The results obtained are expressed in g/L glucose equivalent.

2.3.7. Furfural

The detection of the presence of furfural by a Thermo Finnigan Surveyor with a Aminex 87H 300x7.8 mm column (Biorad) equipped with a UV/vis 280 nm detector. The analysis was performed at 30 °C, with sulfuric acid (10 mN) as eluent, at a flow rate 0.6 mL/min. The standards were prepared in deionized water in concentration of 0.6 ppm to 120 ppm. The analysis was performed at 25 °C, with sodium hydroxide and sodium acetate as eluent, at a flow rate of 1 mL/min.

2.3.8. Alcohol

The detection of the presence of ethanol by a Dionex 3000 chromatograph with an Carbopac MA1 250x4 mm + pre-column (Termo Dionex). The analysis was performed at 30 °C, with sodium hydroxide (480 mM) as eluent, at a flow rate 0.4 mL/min.

2.3.9. Acid Acetic and Formic

The detection of the presence of furfural by a Thermo Finnigan Surveyor with a Aminex 87H 300x7.8 mm column (Biorad) equipped with a UV/vis 210 nm detector. The analysis was performed at 30 °C, with sulfuric acid (10 mN) as eluent, at a flow rate 0.6 mL/min.

2.4. Calculus

2.4.1. Product yield

The product yield ($Y_{P/S,}$ g/g) was determined by the follow equation:

$$Y_{P/S} = \frac{\Delta_P}{\Delta_S} \quad \text{(Equation 2)}$$

where Δ_P is the product produce (g_{EPS}) and Δ_S is the substrate consumed (g_{sugars}) during the assay.

2.4.2. Volumetric productivity

The volumetric productivity (r_P, g/L.d) of the EPS production process was determined using the equation described below:

$$r_P = \frac{dP}{dt} \quad \text{(Equation 3)}$$

where dP corresponds to the variation of concentration of product (EPS, g/L) in a dt interval (hours), that corresponds to the duration of the production assay.

Chapter 3

3. Results and Discussion

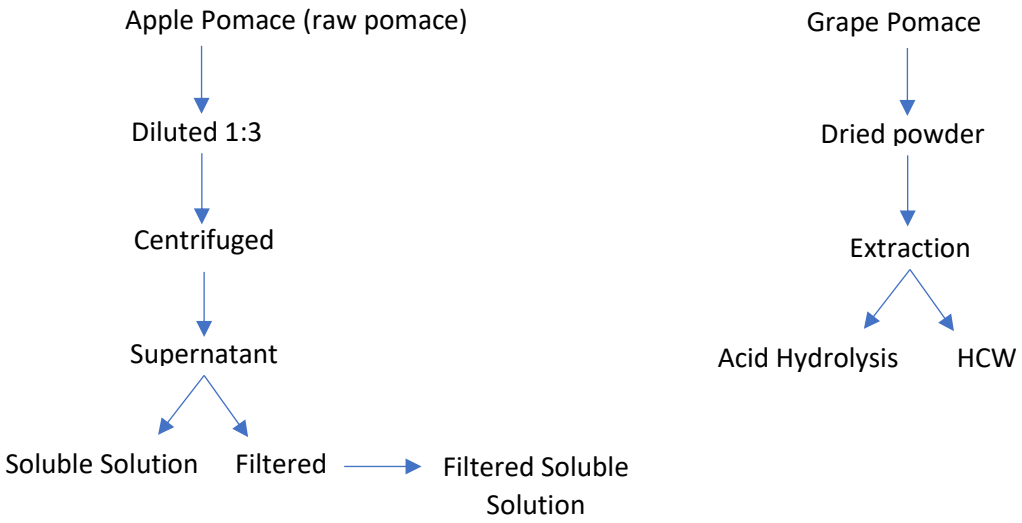

Figure 2: Global scheme of the work

3.1. Production of EPS by *Enterobacter* A47 using apple pomace as substrate

3.1.1. Characterization of apple pomace

The apple pomace was supplied by Sumol + Compal, S.A., the pomace was received on 25 of January, it came in 5 L containers which were divided into ≈ 1.5 L lots and stored at -20 °C until used for the various tests and experiments.

The apple pomace had an orange color and was very thick (Fig. 2), so it had to be diluted for its processing and characterization. Therefore, it was diluted with deionized water in different ratios, namely, 1:2, 1:3 and 1:4, and the mixtures' apparent viscosity, as well as the ability to obtain a clear supernatant upon centrifuging, were evaluated.

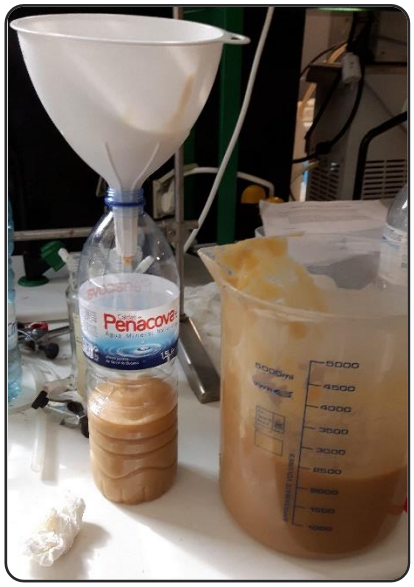

Figure 3: Apple pomace

3.1.1.1. Apparent viscosity

The apparent viscosity was measured for the different ratios. The dilution 1:2 had a shear thinning behavior with the apparent viscosity decreasing as the shear rate increased (Fig. 3). It had an apparent viscosity of 0.183 Pa.s, for a shear rate of 12 s^{-1}, while for the 1:3 and 1:4 dilution ratios the apparent viscosity was 0.004 Pa.s and 0.010 Pa.s, respectively, for the same shear rate. Given these results, the ratio chosen for further studies was 1:3.

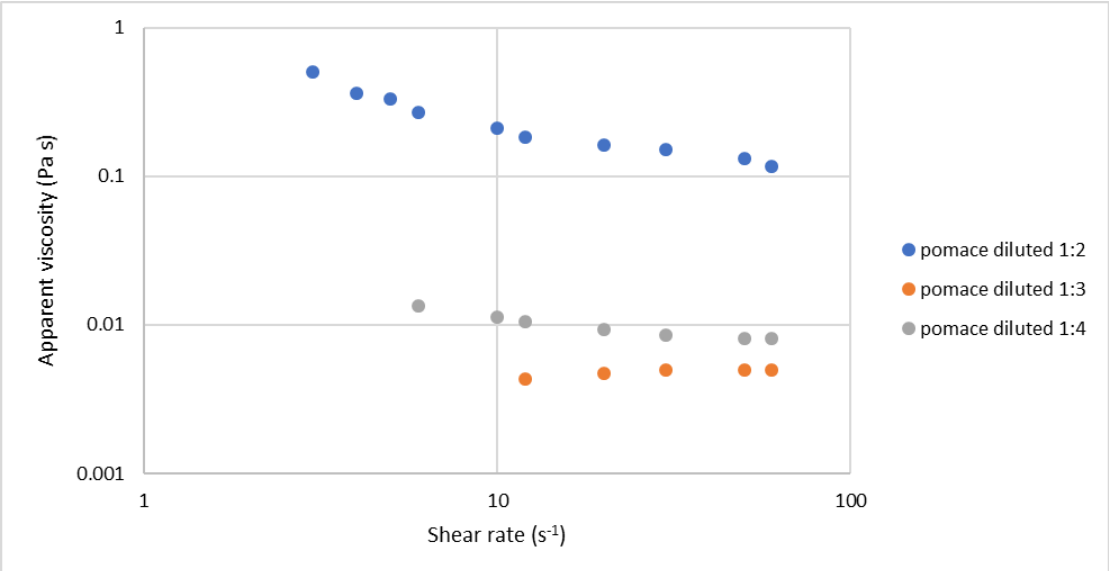

Figure 4: Graphical representation of the shear rate of the apparent viscosity of apple pomace at the different dilutions

With this dilution, the pomace mixture was not very viscous and with only one centrifugation it was possible to separate most of the insoluble material and obtain a clear supernatant (Fig. 4).

Figure 5: Feed solution of apple pomace soluble fraction after autoclaved

3.1.1.2. Physical – Chemical characterization

The physical-chemical characterization of apple pomace was determined (Table 1) in order to know its main components and its suitability to be used as substrate for bacterial cultivation. These components may vary a little from lot to lot, from the year that it is produced and, also depends on the variety of apple that is used to produce the pulp or juice.

The pH of the raw pomace was 3.74 and the conductivity was 276.9 µS/cm (Table 1). Wu et al. (2007) showed that the juice extracted from different varieties of apple have different pH values that may range from 3.59 to 4.16. The raw apple pomace was probably a mixture of different varieties, according to the manufacturer but its pH value was within the range of reported values.

The raw apple pomace showed a density of 22.62 g/cm^{-3}. Kheiralipour et al. (2008) registered a density of 1.48 g/cm^{-3} for apple puree and Pierzynowska-Korniak & Zywica (2004) recorded a density of 1.34 g/cm^{-3} for concentrated apple juice. These values are much lower than the density obtained in this study for the raw apple pomace, which is probably because it was more concentrated, containing a lower water content.

The raw apple pomace had a total dry mass of 18.49 wt.% and its inorganic salts content was only 1.25 ± 0.25 wt.%, for the diluted pomace the content in inorganic salts was 1.65 ± 0.13 wt.%. The insoluble material of the pomace, such as fibers, represented a content of 35.15 g/L, while the soluble material represented a content of 30.02 g/L (Table 1).

The soluble fraction of the diluted pomace had a total nitrogen content of 80.4 mg/L and a content of inorganic salts of 0.60 ± 0.25 wt.% (Table 1). No ammonium was detected. The total sugar present in the pomace supernatant was 42.80 g/L, while the filtered soluble fraction (obtained by filtration of the supernatant using a membrane with a cut-off of 100 000 Da) had a total sugar content of 31.05 g/L. This fraction contained high molecular weight compounds that were present in the soluble fraction and they corresponded to a concentration of 4.96 ± 0.41 g/L in the soluble fraction.

Table 1: Register of the parameters analyzed for the characterization of the apple pomace. Raw apple solution was the original pomace, the soluble fraction was the supernatant resulting from the dilution (1:3) and centrifuge of the raw pomace, the filtered soluble solution was the soluble fraction filtered using a membrane with a cutoff of 100 000 Da (n.d.: not determined).

Parameter	Units	Value
pH	---	3.74
Conductivity	µS/cm	276.9
Apparent density	g/cm^3	22.62 ± 0.43
Total dry mass	%	18.49 ± 0.16
Inorganic salts		
Raw pomace	%	1.25 ± 0.20
Soluble fraction		1.65 ± 0.13
Filtered soluble fraction		0.60 ± 0.25
Insoluble material	g/L	35.15 ± 1.43
Soluble material	g/L	30.02 ± 4.32
Total nitrogen	mg/L	80.4
N	mg/L	n.d.
Total sugar		
Soluble fraction	$g_{Glucose}/L$	42.80
Filtered soluble fraction		31.05

3.1.1.3. Sugar composition

The main simple sugar components detected in all apple pomace samples analyzed were fructose and glucose (Table 2). Both these sugars have already been proven to be suitable carbon sources for cultivation of *Enterobacter* A47 and production of EPS[22,26,27]. The soluble fraction obtained by centrifugation of the diluted apple pomace had a fructose content of 14.95 ± 0.53 g/L and a glucose content of 5.80 ± 0.39 g/L. Besides these two monosaccharides, sucrose was also detected (2.42 ± 0.20 g/L) (Table 2).

When the soluble fraction samples were hydrolyzed, the fructose and sucrose concentrations were decreased (10.80 ± 1.12 g/L, 0.19 ± 0.01 g/L, respectively) indicating that those sugars probably degraded during the hydrolysis procedure. There was also an increase of the glucose concentration (7.51 ± 0.46 g/L) (Table 2), which was probably due to the hydrolysis of some of the oligo- or polysaccharides present in the soluble fraction. In fact, as mentioned above, a high molecular weight fraction (Mw > 12 000 Da) was recovered from the soluble fraction. On the other hand, arabinose and galactose were detected in the samples (2.04 ± 0.26 and 0.08 ± 0.009 g/L, respectively) probably as a result of the hydrolysis of polysaccharides and oligosaccharides present in the apple pomace.

The hydrolyzed raw apple pomace sample had a composition similar to the hydrolyzed soluble fraction sample, in terms of the quantity of fructose (10.27 ± 1.16 g/L) and glucose (7.42 ± 0.12 g/L), arabinose and galactose (2.52 ± 0.20 and 0.35 ± 0.02 g/L, respectively). Moreover, it still had a residual sucrose content (0.51 ± 0.06 g/L).

To evaluate if there was any loss of simple sugars upon sterilization by autoclaving, the soluble fraction was autoclaved and analyzed for its composition. The autoclaved soluble fraction sample was similar to the soluble fraction in terms of the amount of sugars, with only a minor loss of fructose, a slight increase in glucose and sucrose but the overall simple sugars content was similar (Table 2). Similarly, no galactose or arabinose were detected.

Table 2: Comparison of the sugar content in the different samples. (n.d.: not determined)

Sample	Fructose (g/L)	Glucose (g/L)	Galactose (g/L)	Arabinose (g/L)	Sucrose (g/L)	Total (g/L)
Soluble fraction	14.94 ± 0.53	5.80 ± 0.39	n.d.	n.d.	2.42 ± 0.20	23.16 ± 1.08
Hydrolyzed soluble fraction	10.80 ± 0.69	7.51 ± 0.46	0.08 ± 0.009	2.04 ± 0.26	0.19 ± 0.01	20.62 ± 1.39
Hydrolyzed raw apple pomace	10.27 ± 1.16	7.42 ± 0.12	0.35 ± 0.02	2.52 ± 0.20	0.51 ± 0.06	21.09 ± 1.06
Autoclaved soluble fraction	13.59 ± 0.09	6.92 ± 0.15	n.d.	n.d.	1.84 ± 0.84	22.35 ± 1.13
Filtered soluble fraction	15.17	8.17	n.d.	n.d.	n.d.	23.34

Taking into account the results obtained in the characterization of the samples, the soluble fraction obtained with the 1:3 dilution of the apple pomace was selected for the bioreactor

experiments, since this fraction had a relatively low viscosity and a low amount of insoluble material as compared to raw pomace, it presented a good content in sugars, in particular, fructose and glucose, and had a low content of nitrogen. Moreover, the results showed that the soluble fraction could be sterilized by autoclaving and used for cultivation of *Enterobacter* A47 because the loss of simple sugars was not significant.

3.1.2. Bioreator Experiments

3.1.2.1. Apple pomace soluble fraction

3.1.2.1.1. Fed – batch experiment

The experiment was initiated with a batch phase, during which the carbon source was used mainly for cell growth, followed by a fed-batch phase that guaranteed the availability of carbon source. This is considered the best cultivation strategy that maximizes EPS synthesis by *Enterobacter* A47[22].

For this experiment, the maximum specific cell growth rate was 0.29 h^{-1} (Table 3). The value obtained is within the range of values (0.27 h^{-1} to 0.35 h^{-1}) reported for this bacterium, grown in various sources of carbon (glucose, glycerol, xylose, lactose, cheese whey)[6,8,22,26], although it is slightly lower than the values reported for the cultivation in glucose (0.35 h^{-1})[22] or tomato paste (0.27 – 0.33 h^{-1}) which was a substrate rich in glucose and fructose[27].

The maximum CDW (5.20 g/L) was reached at around 11 h, after the batch phase (Fig. 5; Table 3). This value was lower than the one obtained with glucose (8.14 g/L)[22] or tomato paste (10.14 – 13.58 g/L)[27] but still in the range (3.92 – 8.60 g/L) obtained in previous studies with glycerol or lactose[6,8,26]. Due to the limiting conditions of nitrogen imposed, there was no cell growth during the fed-batch phase. There was an apparent decrease in the CDW that was due to the sampling performed, as well as to the feed and NaOH input, which slightly diluted the cellular content.

Table 3: Kinetic and stoichiometric parameters obtained during cultivation of Enterobacter A47 using soluble fraction of apple pomace and filtered solution fraction of apple pomace, and com+arison with different carbon sources (n.a.: data not available)

Substrate	Cultivation Mode	μ_{max} (h^{-1})	CDW (g/L)	EPS_{max} (g/L)	r_p (g/L.d)	$Y_{p/s}$ (g/g)	References
Glycerol	Fed-batch	0.27-0.29	5.70-6.75	7.50-7.97	1.89-2.51	0.10-0.17	6, 8
Glucose	Fed-batch	0.35	8.14	13.40	3.38	n.a.	22
Tomato paste	Fed-batch	0.27-0.33	10.14-13.58	3.99-8.77	1.34-2.92	n.a.	27
Apple pomace – soluble fraction	Fed-batch	0.29	5.20	6.10	5.63	0.16	This study (reactor F)
	Batch	0.27	5.02	4.25	3.43	0.27	This study (reactor M)
Apple pomace – filtered soluble fraction	Fed-batch	0.34	4.19	5.66	3.28	0.22	This study (reactor J)
	Batch	0.33	5.64	4.72	5.67	0.24	This study (reactor L)

At the beginning of the experiment, there was about 0.90 g/L of high molecular weight material that probably corresponded to polysaccharides present in the pomace. EPS synthesis started after about 4 h of cultivation during the batch phase while the culture was growing. When it stopped growing (≈ 11 h), it had already produced 2.30 g/L, but continued to produce EPS, reaching a maximum production of 6.10 g/L at 26 h. After 26 h, there was an apparent reduction that could be due to the degradation of the EPS or, most likely, to the dilution of the broth caused by the input of the feed medium, as happened with the CDW value. The overall volumetric productivity, considering the 0 to 26 h time frame, was 5.63 g/L.d. This value was considerably higher than the values (1.89 – 3.38 g/L.d) reported in previous studies[6,26,22,8] (Table 3). The maximum EPS in all studies was achieved using glucose as carbon source (13.40 g/L), but this value was achieved at the end of 96 h of cultivation, thus corresponding to a volumetric productivity of 3.38 g/L[22].

The bacterium *Enterobacter* A47 has shown a preference in the consumption of the different sugars present in the soluble fraction of the apple pomace, as can be seen in Figure 5.

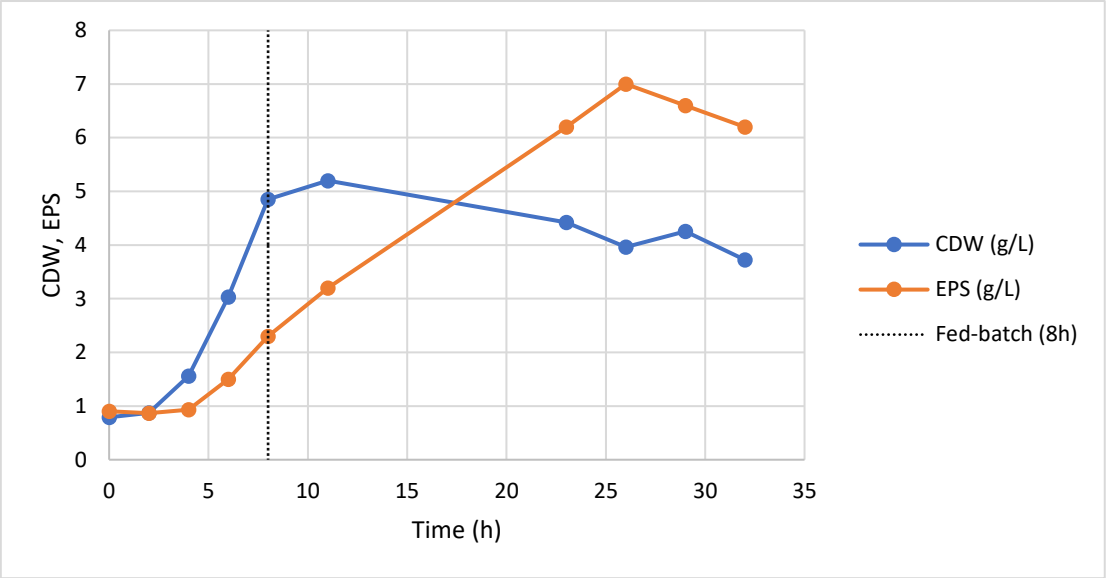

Figure 6: Cultivation profile (CDW and EPS production) during the fed-batch cultivation of *Enterobacter* A47 using the apple pomace soluble fraction as sole substrate.

Glucose consumption was initiated after inoculation and only when the glucose concentration was below 5 g/L the consumption of fructose was initiated. This behavior has already been reported[27] that used tomato paste, a glucose and fructose rich substrate. During the batch phase, all the glucose (10.70 g/L) present in the medium was consumed, as well as most of the fructose (25.63 g/L).

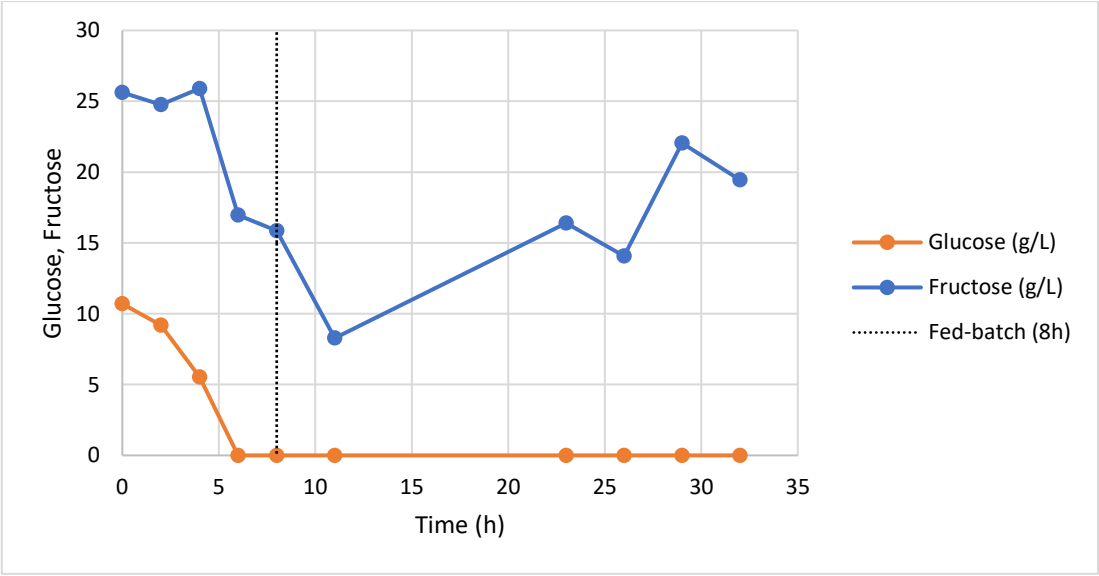

Figure 7: Sugar concentration profile during the fed-batch cultivation of *Enterobacter* A47 using the apple pomace soluble fraction as sole substrate.

After 8 h of the batch phase, the bioreactor started being fed with apple pomace soluble fraction that contained 27.4 g/L of fructose and 13.9 g/L of glucose. The glucose fed was completely consumed upon entering the bioreactor since it was not detected in any sample during the fed-batch phase. Fructose, on the other hand, was not completely consumed.

Between 11 and 23 h, as the culture was not able to consume all fructose from the feed solution, it began to accumulate gradually in the medium. The total sugar consumption was 43.90 g/L, which was composed by 19.19 g/L of glucose and 24.71 g/L of fructose, the overall maximum product yield for this assay was 0.16 g/g, considering the time maximum EPS concentration was reached (26 h) this value was in the range (0.10 – 0.17 g/g) registered in the glycerol assays performed under similar fed-batch conditions (table 3).

The EPS recovered from the broth at the end of the experiment was composed of fucose (40 mol %), glucose (27 mol %), galactose (28 mol %) and glucuronic acid (5 mol %), which similar to the sugar profile reported for FucoPol, although the later typically has a slightly lower fucose content (32-36 mol %) and a higher glucuronic acid content (9-10 mol %).

The soluble fraction of apple pomace seems to be a good substrate for the bacterium, since the main sugars are glucose and fructose, and these have already been shown to be a good source of carbon, capable of being used for bacterial growth, as well as for the production of EPS. Through the use of this substrate, it was possible to obtain a good EPS production, especially because the assays took less than half the time of a standard assay. This substrate may be a good substrate for industrial use since the amount of free sugars present in the medium was high, requiring only a centrifugation step as an upstream procedure.

3.1.2.1.2. Batch experiment

Considering that in the fed-batch experiment described above, the culture initiated the production of EPS still during the batch phase, reaching a production of 2.30 g/L even before the feed was started, a batch assay was done to evaluate the performance of the culture using only the available substrate from the start of the assay, without any further feeding. This way, the dilution effect observed in the apparent reduction of the CDW and EPS concentration at the end of the fed-batch assay was also avoided.

The assay took 29 h (Fig. 7).

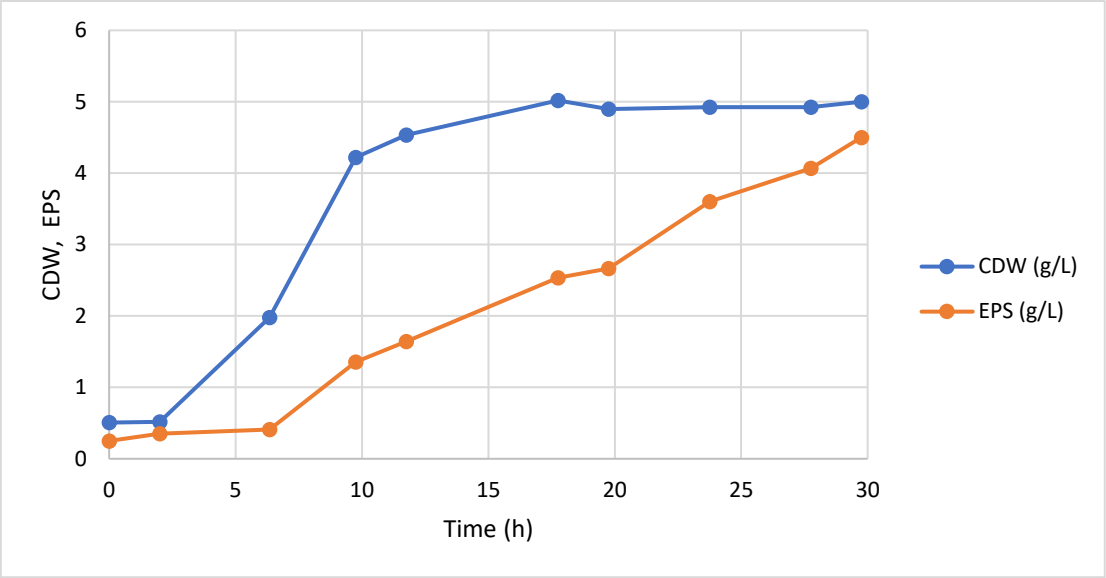

Figure 8: Cultivation profile (CDW and EPS production) during the batch cultivation of *Enterobacter* A47 using the apple pomace soluble fraction as sole substrate.

In this experiment, the culture grew at a maximum specific growth rate of 0.27 h^{-1}, while in the fed-batch process the cell growth little faster (0.29 h^{-1}). Nevertheless, this value was within the range registered for other carbon sources (0.27 – 0.35 h^{-1}), as can be seen in table 3.

For the CDW, the maximum value was 5.02 g/L (Table 3) and was reached after 18 h of cultivation. This value is similar to the one obtained in assay F (5.20 g/L) but it was still in the range registered in other studies (5.70 – 13.58 g/L) as described above.

When the experiment was initiated, there was about 0.25 g/L of high molecular weight material (Fig. 7). This value was lower than the value registered in the fed-batch experiment, which may happen because the pomace was a heterogeneous material and the procedures to obtain the culture medium (dilution, centrifugation) were likely to result in some variability of its composition. As registered in the fed-batch assay, in this assay, EPS production also began while the bacterium was still growing (around 2 h), achieving a production of 1.10 g/L at the final of the growing phase. After that, the bacterium still continued to synthesize EPS, attaining a maximum of 4.25 g/L in 30 h (Table 3). When comparing this value with that of F assay the EPS production was lower, perhaps because the amount of sugar in the medium was lower and the lack of the feed solution that provided more sugars, especially glucose, influenced this decrease in EPS production.

The highest volumetric productivity was registered at 30 h of cultivation, achieving 3.43 g/L.d, this value was in the range (1.34 – 3.38 g/L.d) of the studies with others carbon sources but inferior of the value achieved with the same carbon source (F assay) (5.63 g/L.d) (Table 3).

In the beginning of the assay the glucose concentration was 4.91 g/L and the fructose was 17.65 g/L, the total sugar amount (22.56 g/L) was inferior to the F assay, that was 36.33 g/L, this

difference on the amount of sugar resulted from the fact that the apple pomace has heterogeneous character and the process to obtain the soluble fraction (dilution and centrifuging) influenced the soluble fraction composition, resulting in a medium with less simple sugars.

The concentration profile was similarly to the fed-batch experiment (assay F), glucose was consumed first and only when it reached a limiting concentration fructose started to be consumed (Fig. 8).

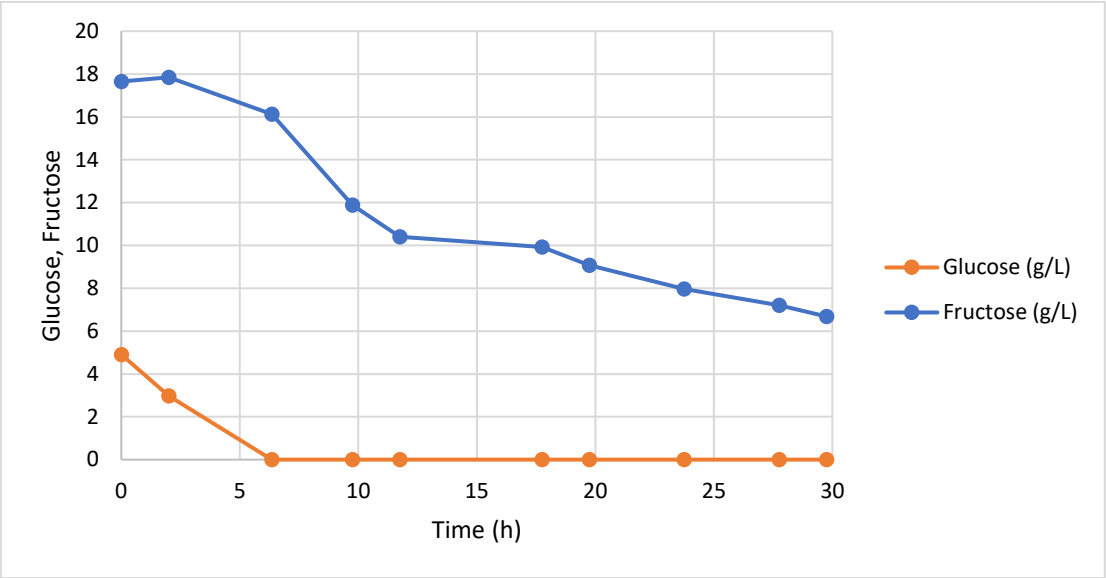

Figure 9: Sugar concentration profile during the batch cultivation of *Enterobacter* A47 using the apple pomace soluble fraction as sole substrate.

Throughout the assay, the bacteria consumed 15.58 g/L of sugars, but at the end there were still some fructose (6.68 g/L) that was not used. The overall product yield in this assay was 0.27 g/g in 30 h, this value is much higher than the one registered in the fed-batch (0.16 g/g), it seems that the culture used more efficiently the fructose in the EPS production. Considering that most of EPS synthesis occurred when glucose was already exhausted in the bioreactor and that only fructose was available, the product yield on a fructose basis was 0.44 g/g, which confirms that the conversion of this sugar into EPS is more efficient than that of glucose (Fig. 8).

For this experiment the EPS was composed of fucose (42 mol %), glucose (27 mol %), galactose (27 mol %) and glucuronic acid (5 mol %), similarly to that of the F assay.

Although it was heterogeneous and caused variability in substrate composition, apple pomace is a good substrate for the cultivation of this bacterium, capable of obtaining quite interesting results from the point of view of cell growth, as well as EPS production. In the assay, there is still a good amount of fructose in the medium, so perhaps the assay should have been extended to obtain a higher EPS production. Comparing the two assays using the soluble

fraction, the fed-batch assay seems to be the best, because with only a further 2 h of cultivation was obtain better EPS yield, although the sugars conversion into EPS was not so efficient.

3.1.2.2. Apple pomace filtered fraction

In these experiments, the apple pomace was filtered using a membrane with a cutoff of 100 000 Da to remove the high molecular weight compounds present in the apple pomace soluble fraction and, hence, to verify if they influence the production of EPS. This resulted in a clear solution since all the particles in suspension were completely removed (Fig. 9). Due to the filtration, the amount of total sugars in this solution (31.05 g/L) was lower than the soluble fraction (42.80 g/L), because the high molecular weight polysaccharides were removed but the content in simple sugars (glucose and fructose) was similar.

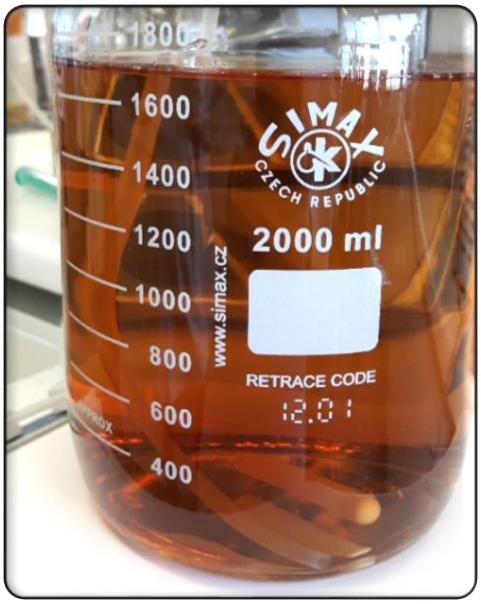

Figure 10: Feed solution of apple pomace filtered soluble fraction after autoclaved

3.1.2.2.1. Fed – batch experiment

For this study, the culture grew with a specific cell growth rate of 0.34 h^{-1}, which is comparable to the value reported for cultivation with glucose as sole carbon source (Table 3). Cell growth stopped after the batch because of the limiting condition of the nitrogen.

The maximum CDW was 4.19 g/L and was reached at the end of the batch phase (8 h) (Fig. 10). Comparing with the other experiments performed with apple pomace and other carbon sources, this value was the lowest reached in all the assays (Table 3). Similarly, to assay F, there was an apparent decline of the CDW throughout the fed-batch phase that was perhaps because

the feed solution and the NaOH were entering the reactor and diluting the broth, concomitant with the withdrawal of samples.

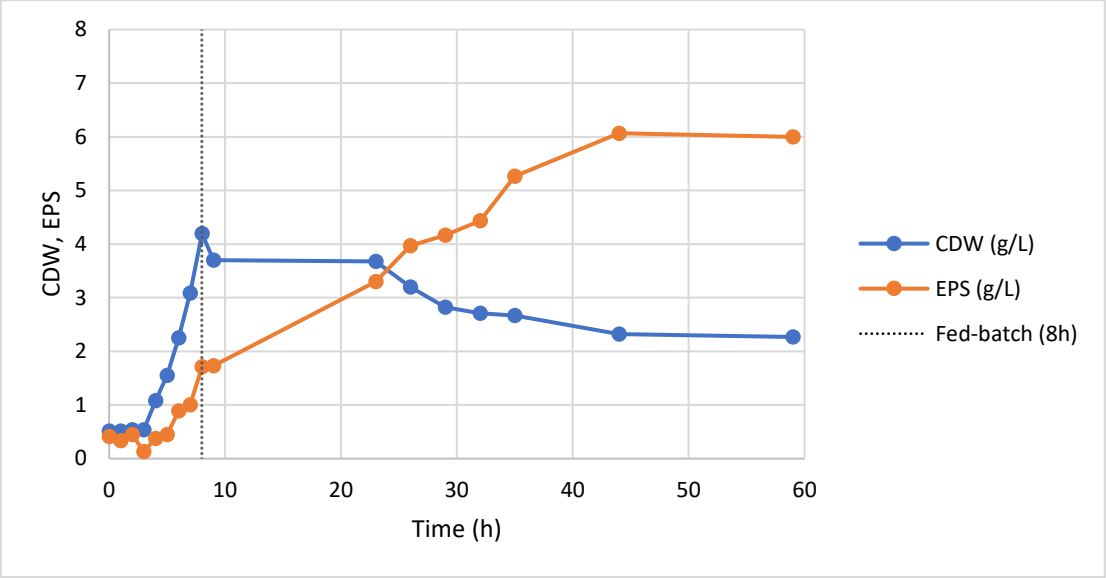

Figure 11: Cultivation profile (CDW and EPS production) during the fed-batch cultivation of *Enterobacter* A47 using filtered soluble fraction of the apple pomace.

Even the medium being filtered, it contained about 0.33 g/L of high molecular weight material (>12 000 Da), which was probably a fraction of polysaccharides smaller than the UF membrane cut-off (100 000 Da) and was passed to the medium.

The production of EPS was initiated during the batch phase, after around 3 h of cultivation, when the cell growth had ceased (8 h). At that time, the culture had already produced 1.30 g/L of EPS. At the end of the batch phase in F assay, the culture had already produced more EPS (2.30 g/L), but the growth phase was longer (11 h). During the fed-batch phase, the culture continued to produce EPS reaching a maximum production of 5.66 g/L in 44 h (Fig. 10). At that time, EPS production seem to slow down or the medium to diluted. Contraries of what happens in the F assay, in this experiment the bacterium still produces EPS after the 25 h, this may be happening because of the absence of suspended particles, that does not put the bacterium in so high-stress, behaving more like a standard assay.

In this assay, the maximum volumetric productivity was achieved at 26 h with a value of 3.28 g/L.d (Table 3). This value was a lower when compared with the one obtained in assay F (5.63 g/L.d), but still inside of the range (1.34 – 3.38 g/L.d) reported for other carbon sources (Table 3).

When the assay was started, there were 11.09 g/L glucose and 22.10 g/L fructose in the medium, the amount of glucose was higher than in the F and M experiment (10.70 and 4.91 g/L, respectively), but the total amount of sugar at the start (33.19 g/L) was lower than for the F

assay (36.33 g/L), the M assay had the lowest amount of initial sugar with only 22.56 g/L (Fig. 11). This assay presents a sugar concentration profile similar to that of assay F, in which glucose is the first sugar to be consumed. When the glucose dropped to below 5.0 g/L, the bacteria began to consume the fructose, this behavior was equal to observed in the F assay. When the culture reached 8 h, the feed solution, containing 14.7 g/L glucose and 27.3 g/L fructose, started to be added to the bioreactor, the presence of glucose was not detected after the beginning of the feeding phase, which means that all glucose from the feed solution was consumed as it entered the reactor (Fig. 11). Until around 23 h of cultivation, the concentration of fructose decreased until it reached 6.57 g/L, after which fructose started to accumulate progressively in the medium, this shows that the rate of fructose consumption was lower than the rate of fructose intake in the reactor (so it accumulated). A similar trend was observed in assay F.

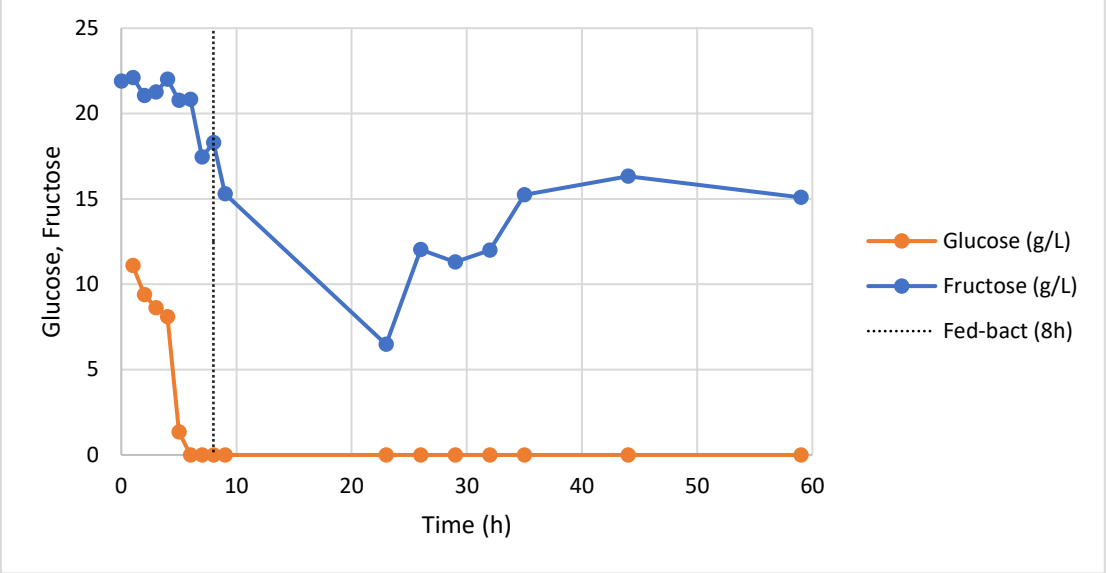

Figure 12: Sugar concentration profile during the fed-batch cultivation of *Enterobacter* A47 using the filtered soluble fraction of the apple pomace as sole substrate.

In the end, the total amount of sugar that was consumed was 40.4 g/L, composed of 12.97 g/L of glucose and 16.34 g/L of fructose, in the F assay the total consumption was slightly higher (43.90 g/L). The maximum product yield was 0.22 g/g in 44 h, this value was on average observed for the apple pomace assays (Table 3), comparing this value with the other fed-batch (F assay) using a non-filtered medium (0.16 g/g) was obtained a superior value.

The resulting EPS was composed of fucose (42 mol %), glucose (28 mol %), galactose (26 mol %) and glucuronic acid (4 mol %), when compared to the F and M assay, the composition of the EPS did not change much, showing that the use of the filtered apple pomace fraction had no significant impact on the polymer's composition.

It seems that using a filtered medium in this type of cultivate (fed-batch) was not the best method of cultivation, since both the productivity and the EPS production are inferior to F assay (soluble fraction), despite cell growth and yield that were slightly higher than those of F.

3.1.2.2.2. Batch experiment

This assay was performed using a solution filtered as assay J, but in batch mode, in order to compare the production of EPS with the M assay and to evaluate the interference of the polysaccharides in their production. Comparing this batch assay with the M assay (30 h), this assay took almost the same time (26 h).

The culture grew at a maximum specific growth rate of 0.33 h^{-1} (Table 3). As expected, this value is similar to the one obtained in assay J (0.34 h^{-1}).

A maximum CDW (5.64 g/L) was reached after 12 h, being maintained practically unchanged until the end of the assay (Fig. 12).

Similarly to assay J, there was a content of 0.68 g/L of high molecular weight material at the beginning of the run. The culture initiated EPS synthesis after 2 h of cultivation, achieving 3.27 g/L in the end of the growing phase, while a maximum production of 4.72 g/L was attained at 20 h. The EPS production value was slightly higher than the one recorded in the assay M (4.25 g/L) that was done with unfiltered apple pomace soluble fraction. The overall volumetric productivity, considering the time frame of 0 – 20 h, was 5.67 g/L.d (Table 3). However the maximum value for the volumetric productivity was observed at 12 h: 8.11 g/L.d, which is the highest value registered for FucoPol production.

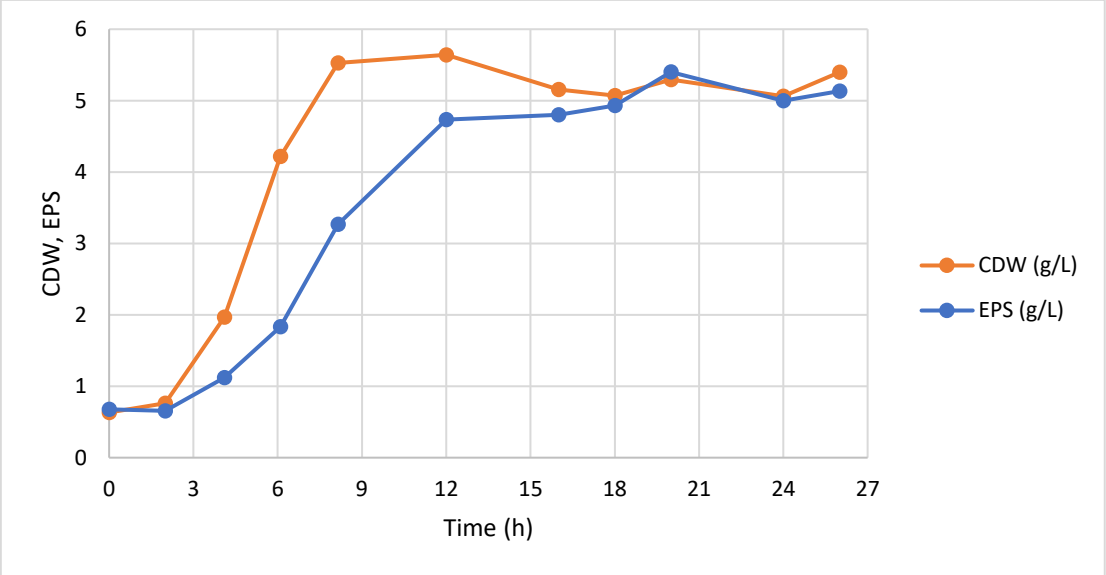

Figure 13: Cultivation profile (CDW and EPS production) during the batch cultivation of *Enterobacter* A47 using filtered soluble fraction of the apple pomace.

This sugar concentration profile is slightly different from the profiles observed above. The glucose was still the first sugar that was consumed (4.11 g/L), when the culture consumed all the glucose, started to use the fructose (25.67 g/L) and in this case the fructose was totally consumed within 20 h (Fig. 13), this may happen because of the variability of apple pomace.

The quantity of sugar the beginning of the assay was 4.11 g/L of glucose and 26.57 g/L fructose since all of the sugar amount was used by the bacterium, the total was 29.78 g/L.

In this assay, contrary of what happens in the previous assays all the sugar present in the medium was consumed by the bacterium so for that reason the EPS production end up at 20 h of cultivation. Likewise, as in the M assay, basically, all the EPS produced by the bacterium was produced using just the fructose as the carbon source.

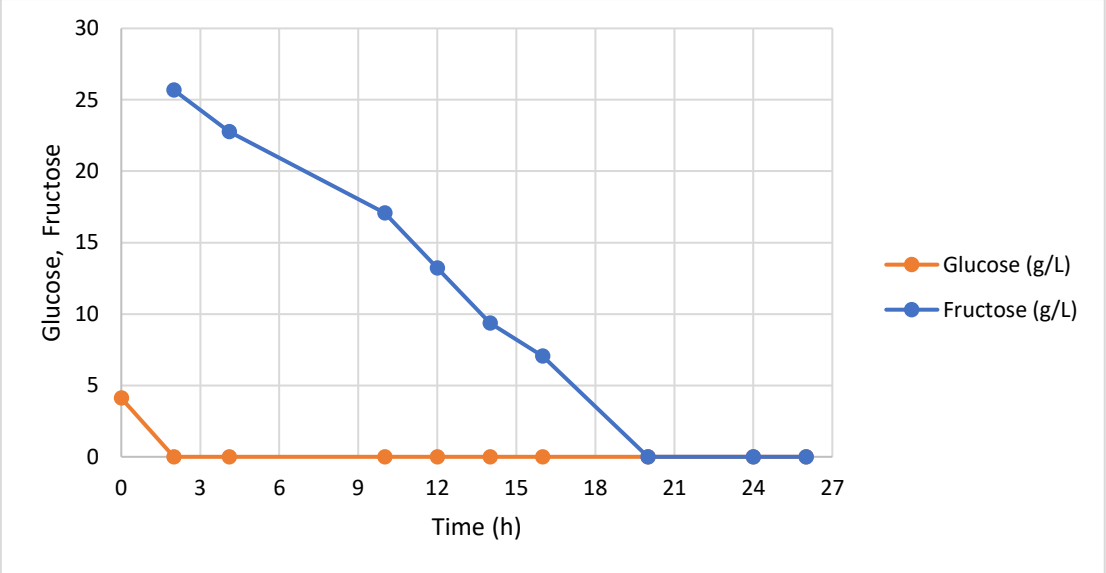

Figure 14: Sugar concentration profile during the batch cultivation of *Enterobacter* A47 using the filtered soluble fraction of the apple pomace as sole substrate.

The overall maximum product yield in this assay was 0.24 g/g in 12 h, in comparison with the M assay that has the same form of cultivation (batch mode) the value was inferior (0.27 g/g), but when compared with the J assay, that has the same medium the value was similar (0.22 g/g).

The final EPS was composed of fucose (39 mol %), glucose (30 mol %), galactose (27 mol %) and glucuronic acid (4 mol %). From all the assays using apple pomace, this was the assay with the lower fucose amount and higher glucose amount, the galactose and glucuronic acid were still in the range obtained for the other assays. Comparing with the standard assay with glycerol, the amount of fucose was superior, the glucose was in the range and the galactose was slightly above of the range, while, the glucuronic acid was lower.

Though the solution filtered as a culture medium for fed-batch production did not work as expected (J assay), the same cannot be said for the batch production which obtained the highest

productivity using the apple pomace, good cell growth, and yield. EPS production was slightly low perhaps because the substrate was exhausted, but the maximum volumetric productivity was high. From an industrial point of view, centrifugation and/or filtration are commonly used procedures, easy to implement and not expensive. So, the use of the filtered apple pomace fraction as sole substrate for the production of FucoPol seems promising.

3.1.2.3. Conclusion

This study showed that apple pomace can be efficiently used as a substrate for *Enterobacter* A47 cultivation, achieving good results in cell growth and FucoPol production. The results indicate that fed-batch mode still the best way to produce high EPS concnetrations with this bacterium, but higher volumetric productivity was reached using the batch mode. According to the results, the best medium between soluble fraction and filtered fraction depends on the mode that was used. The soluble fraction was the best medium for fed-batch mode and the filtered solution was the best medium when was cultivated using the batch mode. The next steps for this study are to try to obtain the same sugar amount in the beginning of the assay and to find a way to contradict the heterogeneity of the apple pomace.

In this study it was possible to reduce the time of obtaining the EPS to less than half, still increasing the productivity and the yield of the same.

3.2. Production of EPS by *Enterobacter* A47 using grape pomace as substrate

The grape pomace was supplied by a Portuguese wine producer, the grapes came from the Alentejo region and were part of the last wine production (2016), came in wet form (Fig 14: A), it was divided into zipped bags (+/- 600 g). The bags were then stored in the freezer at -20 °C until used in the various tests.

3.2.1. Characterization of grape pomace

Figure 15: A- Grape pomace as supplied; B- Grape pomace dried in the oven; C- Grape pomace powder

Gape pomace was constituted by residues left by destemming and alcoholic fermentation, such as skins, seeds and stem pieces (Fig. 13: A). For a more homogenous characterization of grape pomace, it was dried in an oven overnight and then triturated (Fig. 14: B and C).

3.2.1.1. Physical – Chemical characterization

3.2.1.1.1. Granulometry

The granulometry was performed using the grape pomace powder, in order to evaluate the particle size distribution (Fig. 15).

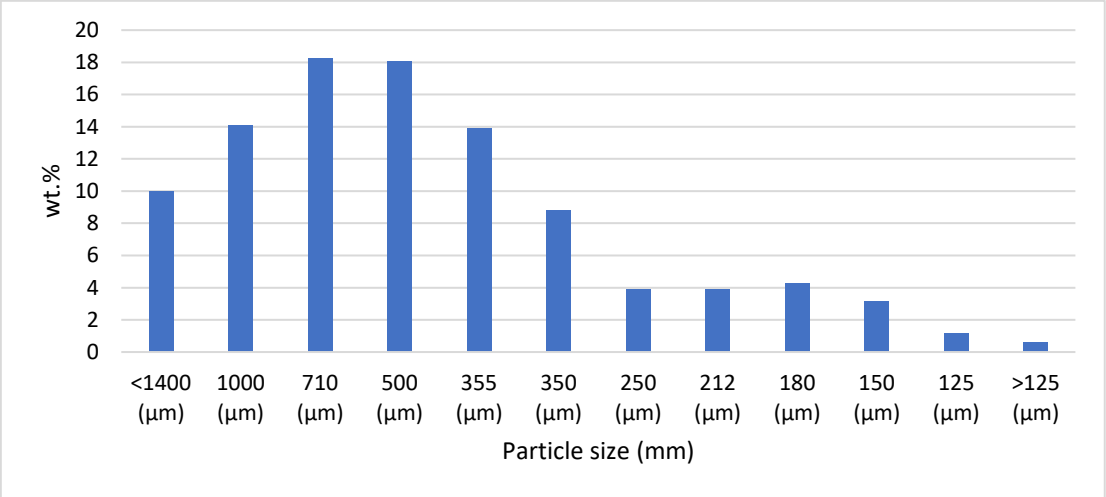

Figure 16: Particle size distribution of the grape pomace powder.

As shown in Figure 15, the grape pomace powder was composed of particles of varying sizes. Most of the particles (> 80 wt.%) had a size above 350 µm. The grape pomace powder had a minor content (1 wt.%) of particles with a size below 125 µm, these results show that the powder

was relatively homogeneous, with most of the particles (64 wt.%) having a size between 1000 and 355 µm.

3.2.1.1.2. Subcritical water extract

In order to analyze the composition in sugars and their distribution, fractions were collected at different temperatures throughout the assay (Table 4).

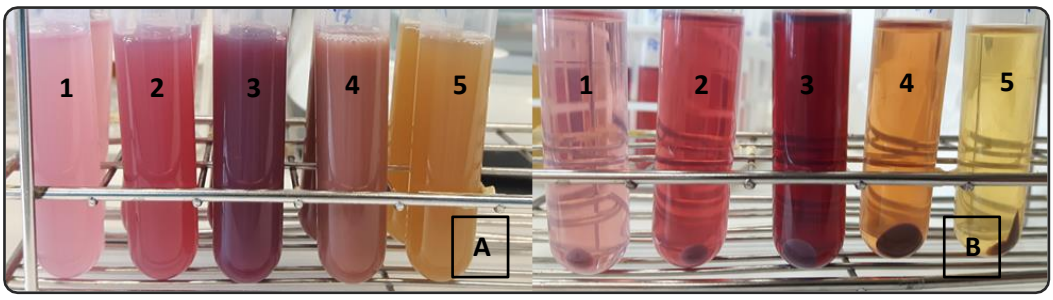

Figure 17: A- Extracts of subcritical water extraction in different temperatures; B- Extracts of subcritical water extraction in different temperatures, centrifuged.

In the beginning of the assay, 60.68 g of grape pomace powder was placed inside the reactor, after the extraction the residue was dried in the oven and was obtain 33.70 g of the grape powder, the overall extraction yield was 44.46 %.

Table 4: Register of the parameters analyzed for the characterization of the grape pomace soluble fraction obtained by subcritical water extraction

Fraction	1	2	3	4	5
Temperature (°C)	0-49	50-99	100-149	150-179	180
pH	3.06	3.58	3.52	3.58	3.77
Conductivity (mS/cm)	3.61	3.65	3.20	1.70	1.25
Insoluble material (g/L)	0.77	0.85	21.67	8.53	0.43
Soluble material (g/L)	42.47	36.03	37.60	31.92	10.03
Total nitrogen (mg/L)	87.1	86.0	73.0	125	99.6
N (mg/L)	43.52	43.57	32.30	90.09	75.26
High molecular weight material (g/L)	0.58	0.90	3.38	9.29	0.37
Sugar compositionm (g/L)					
Trehalose	0.82	0.55	0.41	0.05	n.d.
Rhamnose	0.02	0.01	0.01	0.06	n.d.
Arabinose	0.02	0.02	0.04	0.25	n.d.
Galactose	0.03	0.02	0.03	0.10	0.05

Fraction	1	2	3	4	5
Glucose	2.55	2.04	1.61	0.36	0.09
Fructose	3.01	2.24	1.64	0.26	0.00
Xylose/Mannose	n.d.	n.d.	n.d.	0.11	0.04
Galacturonic acid	0.02	0.01	0.00	0.05	0.00

This characterization was made using an extract grape pomace solution using subcritical water. The pH was measure for all the fraction and as showed in table 4 does not change too much, with the first fraction having the lowest pH and the fifth fraction the highest pH. In the conductivity, the value was higher in the two-initial fraction, decreasing until the last fraction (Table 4). The total sugar present in the HCW extract was 5.87 g/L and the content in inorganic salts was 9.19 ± 0.74 %.

The insoluble material (21.67 and 8.53 g/L) and the high molecular weight material (3.38 and 9.29 g/L), has the highest values in the same two fractions (100-149 °C; 150-179 °C), which means that they may be related, this insoluble material may be cellulose, hemicellulose and lignin of small dimensions that were not hydrolyzed and eventually was removed by the extraction (table 4). The amount of soluble material in the first fraction was 42.47 g/L and decreased during the test ending with 10.03 g/L, which means that the soluble material, such simple sugars, were only extracted from grape pomace and no lignocellulosic residue was hydrolyzed.

For the nitrogen and total nitrogen presented similar results, the higher amounts in the two were detected in the fraction of 150 – 179 °C, probably because it was in this fraction that more protein was degraded.

Glucose and fructose are the principal sugars detected in the soluble fractions, the first two fractions are where the greatest amount was found (glucose: 2.55 – 2.04 g/L; fructose: 3.01 – 2.24 g/L), and this decreased along the fractions, showing a profile equal to that of the soluble material, which also helps to justify that there was no hydrolysis of the lignocellulosic components present in the grape. The total amount of glucose was 6.65 g/L and fructose was 7.15 g/L, trehalose showed an equal profile to the glucose and fructose, having a total amount of 1.83 g/L (table 4). In addition to these three sugars, others were detected, such as arabinose, rhamnose, galactose, xylose/mannose and galacturonic acid but in residual concentrations, unlike the sugars mentioned above, these slightly increase the concentration in the third fraction which indicates that there was hydrolysis of some material but was not very significant. The overall sugar concentration in the soluble fraction was 16.80 g/L.

When the soluble fraction centrifuged like above and hydrolyzed, the concentration of trehalose decreased, since the trehalose is a disaccharide of glucose, when hydrolyzed the trehalose became glucose. For glucose, rhamnose, arabinose, galactose and galacturonic acid, the sugar concentration increased a little, this raise was probably due to the hydrolysis of some insoluble lignocellulosic material present in the fraction. Xylose/Mannose was the only sugar that increased significantly with hydrolysis. It was not possible to confirm whether the peak in the HPLC analysis corresponded to xylose or mannose (table 5). As trehalose, fructose also reduced the concentration maybe because of some degradation during the hydrolysis procedure. The overall sugar concentration of the soluble fraction increased a little when hydrolyzed, attained 20.36 g/L.

Table 5: Sugar composition of the hydrolyzed soluble fractions obtained by subcritical water extraction. (n.d.: not determined)

Fraction	1	2	3	4	5
Threalose	n.d.	0.14	0.09	0.03	n.d.
Rhamnose	n.d.	n.d.	0.13	0.15	0.02
Arabinose	n.d.	n.d.	0.66	0.61	0.02
Galactose	0.10	0.10	0.22	0.46	0.09
Glucose	3.50	2.47	2.11	1.95	0.32
Fructose	2.07	1.15	0.86	0.18	n.d.
Xylose/Mannose	0.08	0.07	0.22	1.13	0.42
Galacturonic acid	0.12	0.09	0.21	0.27	n.d.

The hydrolyzed raw fraction, has a composition similar to the hydrolyzed soluble fraction, in terms of the quantity of glucose, fructose, arabinose, xylose/mannose and galacturonic acid (table 6). The amount of trehalose increase a little when compared with the hydrolyzed soluble fractions but still inferior to what achieved in the soluble fractions, rhamnose seem to decrease when compared with the results above. In this case the overall sugar concentration of the raw hydrolyzed fractions was 20.64 g/L.

Table 6: Sugar composition of the hydrolyzed raw fractions obtained by subcritical water extraction (n.d.: not determined).

Fraction	1	2	3	4	5
Trehalose	0.29	0.14	0.09	0.02	n.d.
Rhamnose	n.d.	n.d.	n.d.	0.16	n.d.
Arabinose	n.d.	n.d.	0.68	0.61	n.d.
Galactose	0.10	0.10	0.25	0.48	0.08
Glucose	3.43	2.44	2.33	2.16	0.29

Fraction	1	2	3	4	5
Fructose	2.08	1.13	0.88	0.16	n.d.
Xylose/Mannose	0.08	0.05	0.26	1.14	0.39
Galacturonic acid	0.12	0.08	0.26	0.34	n.d.

The sugar composition of the extract showed some sugars that were never tested for cultivation of *Enterobacter* A47, namely arabinose, rhamnose, mannose, trehalose and galacturonic acid. Hence, it was considered interesting to test whether such a sugar mixture would be suitable for EPS production by this bacterium.

Looking for all the results that came from the grape pomace characterization, for the subcritical water experiment, the soluble extract was chosen because the difference of the total amount of sugar in the samples was not significant, have a higher trehalose content, this sugar could give interesting properties to the EPS and a more green process. For the acid hydrolysis, the content of soluble polysaccharides was a good indicative for reaching a good amount of simple sugars.

3.2.1.1.3. Grape pomace hydrolysate

The grape pomace hydrolyzate was prepared using the grape marc powder, the same as that used for subcritical extraction. The grape marc powder was mixed with deionized water (Fig. 17: A) and autoclaved (Fig. 17: B), after which the solution was centrifuged and filtered. Subsequently, H_2SO_4 was added to the solution and then was again autoclaved (Fig. 17: C).

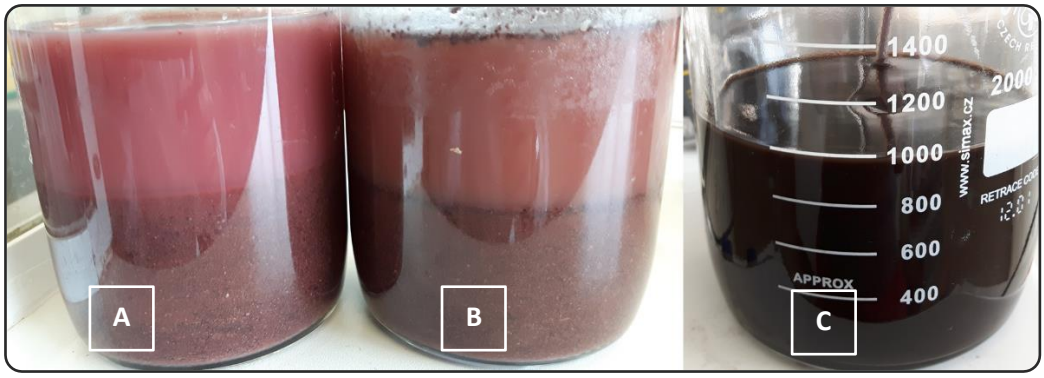

Figure 18: A- mixture of grape pomace powder with deionized water; B- mixture A autoclaved; C- hydrolyzed solution with pH=6.5.

The pH of the final solution was around 2 and had to be set at pH=6.5 before the assay began by the addition of NaOH. The sugars present in the hydrolyzate were the same as in the soluble fraction: arabinose (1.01 g/L) and glucose (2.23 g/L) were those with the highest amount,

followed by fructose (0.30 g/L), galactose (0.18 g/L) and rhamnose (0.04 g/L), xylose/mannose (0.07 g/L), trehalose (0.06 g/L), and galacturonic acid (0.03 g/L) were the sugars detected in less quantity, respectively. The total sugar concentration was 9.27 g/L, the presence of high molecular weight compounds was also detected, but their weight was not determined.

3.2.2. Bioreactor experiments

3.2.2.1. Grape pomace – Subcritical Water

3.2.2.1.1. Fed – batch experiment

In this experiment, the growth of the bacterium occurred at a specific cell growth rate of 0.14 h^{-1} (table 6). Comparing this value with the others achieved in this study and others (0.27 – 0.36 h^{-1}), the value was too low, this may have happened because of the complexity of the medium and the lower content in simple sugars, which probably influenced the cell development.

Table 7: Kinetic and stoichiometric parameters obtained during cultivation of *Enterobacter* A47 using grape pomace HCW extract and acid hydrolysate, and comparison with different carbon sources (n.a.: data not available; n.d.: not determined)

Substrate	Cultivation mode	μ_{max} (h^{-1})	CDW (g/L)	EPS_{max} (g/L)	r_p (g/L.d)	$Y_{p/s}$ (g/g)	References
Glycerol	Fed-batch	0.27-0.29	5.70-6.75	7.50-7.97	1.89-2.51	0.10-0.17	6, 8, 83
Glucose	Fed-batch	0.35	8.14	13.40	3.38	n.a.	22
Grape pomace-subcritical extraction	Fed-batch	0.14	4.20	0.63	n.d.	n.d.	This study
Grape pomace – acid hydrolysis	Fed-batch	0.06	9.19	4.32	4.80	0.51	This study

The CDW reached a maximum at 19 h, having a concentration of 4.20 g/L, long after the batch phase ended (Fig. 18, Table 7). This value was similar to the one obtains in the J assay (filtered apple pomace, fed-batch mode) that was 4.19 g/L, comparing with other studies (5.70 – 13.58 g/L) using glycerol, tomato paste or glucose, the value was much lower. The most likely cause for this lower CDW was that the substrate concentration was lower.

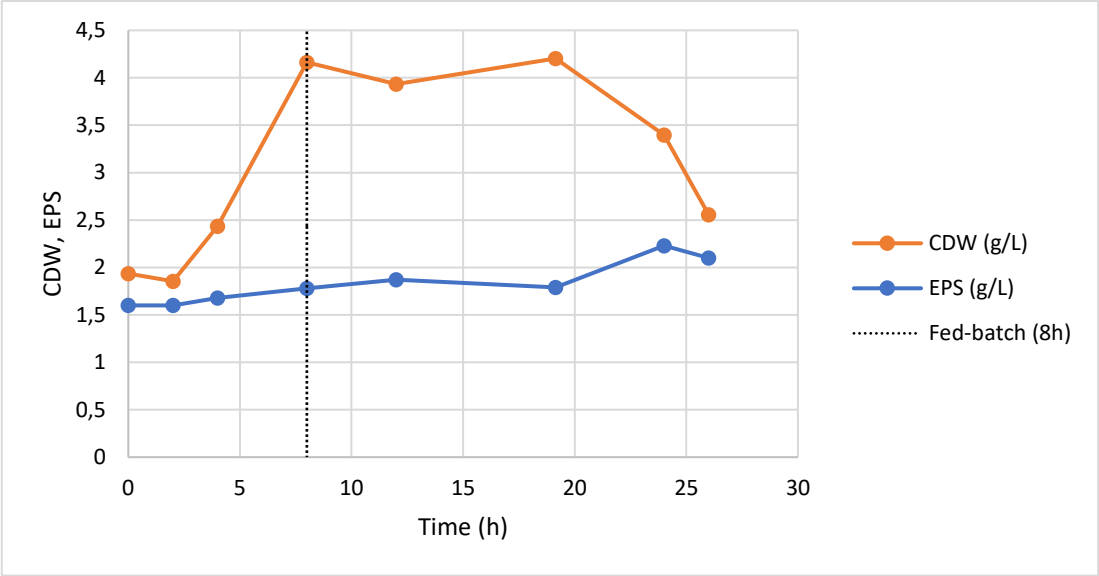

Figure 19: Cultivation profile (CDW and EPS production) during the fed-batch cultivation of *Enterobacter* A47 using soluble fraction of the grape pomace.

Present in the medium was a high quantity of high molecular height substances (1.60 g/L) that came from the subcritical water extract. At the end of the batch phase (8 h) 1.78 g/L of high molecular weigth material was detected in the broth, indicating that the culture had initiated EPS synthesis, although very low (0.28 g/L) (Fig. 18). During the fed-batch phase, practically no EPS synthesis has occurred, and the slight increase observed in the high molecular weight fraction detected was probably due to the accumulation of material present in the feeding solution. This was probably due to the very reduced amount of simple sugars available in the HCW extract.

As similar to what happened in studies using multiple sugars as carbon source, *Enterobacter* A47 had showed a preferential consumption of some sugars over others (Fig. 19).

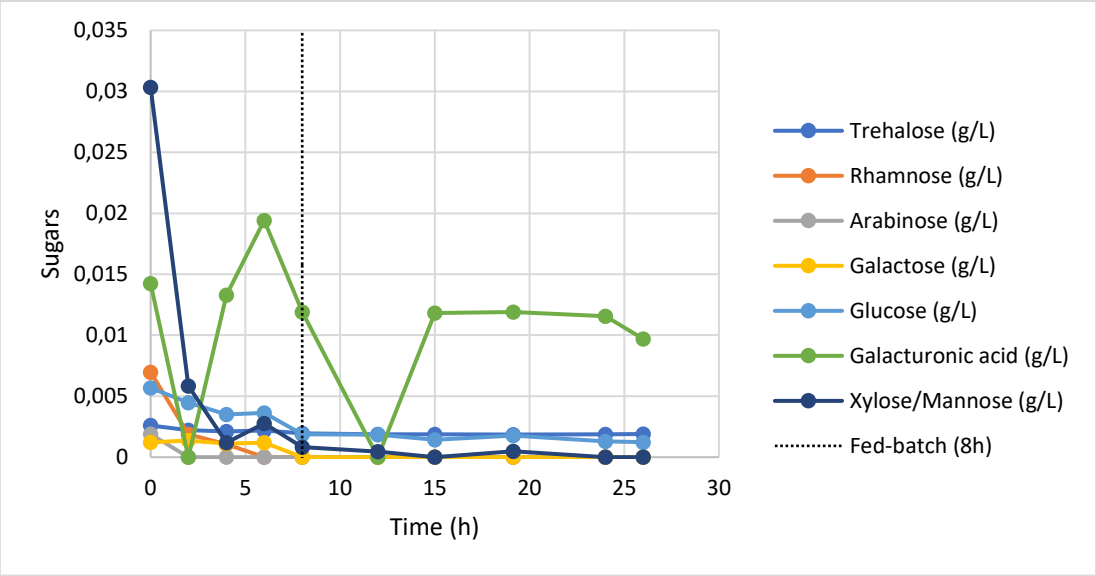

Figure 20: Sugar concentration profile during the fed-batch cultivation of Enterobacter A47 using the soluble fraction of the grape pomace as sole substrate

The sugars that were consumed first were xylose/mannose (0.003 g/L), arabinose (0.002 g/L), rhamnose (0.006 g/L) and glucose (0.006 g/L), the arabinose was completely consumed in the first 2 h, the glucose was consumed very slowly. At 6 h after the beginning of the assay, was given more medium to try to stimulate cell growth and the production of EPS, this made that some sugar like, glucose, galactose and xylose/mannose, raise a little bit the concentration. After 8h, only xylose/mannose was being consumed, ended at 15 h. Trehalose was detected in the medium but apparently was not consumed by the bacterium, the total sugars consumption was 0.1 g/L.

The sugar monomer profiler from the samples extracted at the end of the assay revealed the presence of glucose (27 mol %), galactose (11 mol %), mannose (37 mol %) and arabinose (25 mol%), which confirmed that the bacterium did not produce any EPS and these monomers probably came from the grape pomace HCW extract.

For the solution of grape pomace resulting from extraction with subcritical water to be used as a culture medium, it was necessary that the concentration in sugars was much higher. It seems that in the subcritical water extraction the lignocellulosic material was not hydrolyzed and just was removed the simple sugars that was still in the grape pomace. Perhaps it was necessary an extraction with higher temperature. It was also detected the presence of 5HMF (0.02 ± 0.07g/L), alcohol (1.66 ± 0.36 g/L), formic acid (4.33 mM) and acetic acid (2.24 mM) which despite being inhibitors of cellular growth seems not to have influenced much, because the bacterium still grew.

3.2.2.2. Grape pomace – Acid Hydrolysis

3.2.2.2.1. Fed – batch experiment

In the beginning of the assay the pH was very low (under pH=2, minimum that the system could read) due to the acid hydrolysis, so the system was supplied with NaOH pellets (a sodium content of 47.86 ± 4.68 g/L was attained in the medium), until the pH was around to 6.5.

In this experiment, the bacteria grew at a rate of 0.06 h^{-1}, the value is very low when compared to other studies (Table 7). This delay in bacterial growth, was may be due to the fact that the initial conditions were not ideal (too much sodium salts) and the bacteria took more time to adapt and grow.

Nevertheless, the CDW reached a maximum of 5.81 g/L during the batch phase, after the batch phase the CDW continuous to rise until a maximum of 9.19 g/L at 27.6 h (Table 7). The concentration of CDW dropped a little at the end of the assay, this can happen due to several facts, such as, the broth started to be diluted (feed and NaOH was entering), the cell concentration decreased due to the samples that were withdrawn, or due to the fact that in the beginning there were particles in the broth that could influence the CDW and, in the end, the concentration of these particles decreased (Fig. 20).

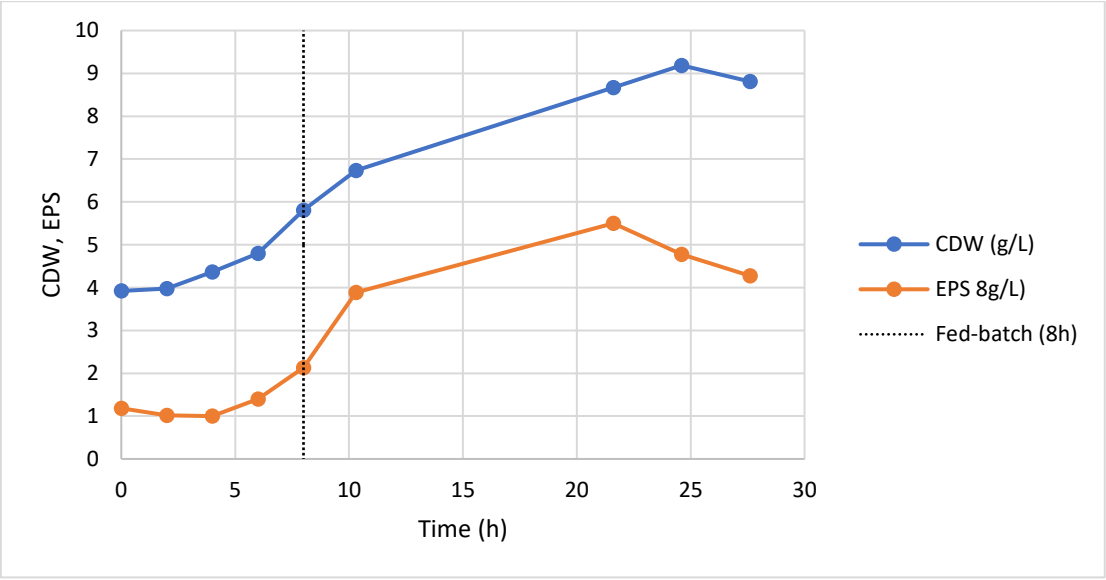

Figure 21: Cultivation profile (CDW and EPS production) during the fed-batch cultivation of *Enterobacter* A47 using a hydrolyzed solution of the grape pomace

In the medium were particles that came from the hydrolysis and were not removed in the filtration, for this reason at the beginning, they were about 1.18 g/L of high molecular weight material, although this apparently did not influence the production of EPS.

Contrary to what happens normally, EPS production only started in the fed-batch phase after 10 h, probably due to the delayed cell growth. Nevertheless, the maximum production was 5.50 g/L at 22 h of cultivation. In the end of the assay, the EPS production dropped perhaps because polymer synthesis slowed down due to substrate limitation or because of the continuous feed phase that diluted the medium. Comparing the production with other studies, this production value was lower than the others but this concentration was achieved in just 22 h (Table 7). For maximum volumetric productivity, there is a maximum value at 22 h of 4.80 g/L.d. This value is higher than the ones registered for glycerol or glucose (Table 7).

Similar to what happened in N assay, the bacterium has shown a specific order of consumption of sugars (Fig. 21).

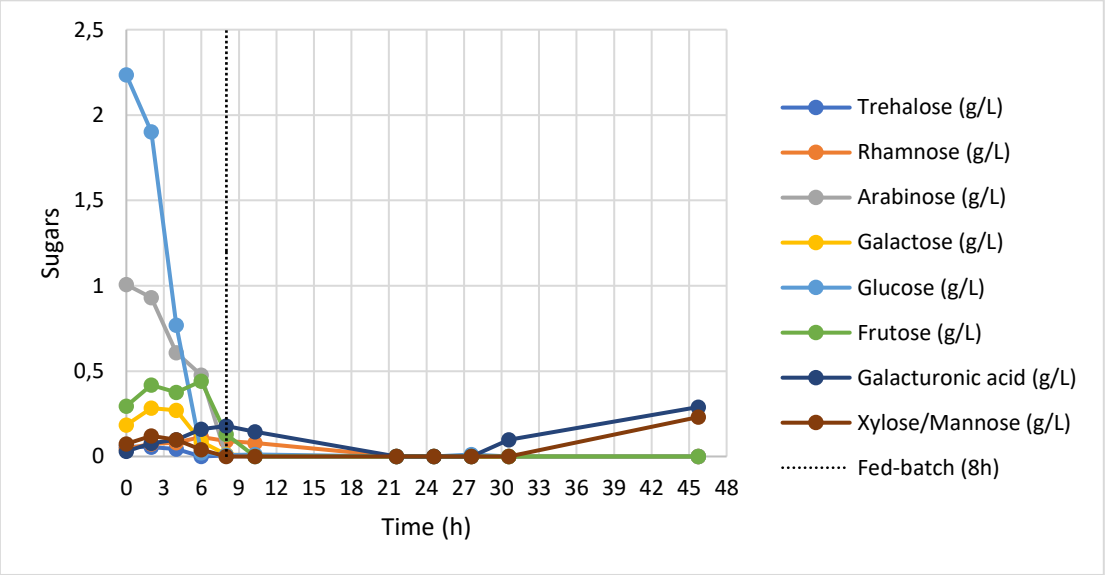

Figure 22: Sugar concentration profile during the fed-batch cultivation of Enterobacter A47 using hydrolyzed solution of the grape pomace as sole substrate

In this consumption profile, the first sugar to be consumed was the glucose, this was already expected, because, in the assay with more than one sugar, *Enterobacter* A47 started to consume glucose (2.23 g/L) in the first place. Arabinose (1.01 g/L) was consumed at the same time as glucose but at a slower rate. When the glucose and arabinose concentrations dropped to below 0.77 g/L and 0.02 g/L, respectively, the bacteria started using the available galactose (0.27 g/L), xylose/mannose (0.12 g/L) and trehalose (0.04 g/L). After that, it was the fructose (0.44 g/L) that was used, the final sugars that were used were rhamnose (0.11 g/L) and galacturonic acid (0.18 g/L). After 22 h all the sugar from the feed solution were consumed immediately (Fig. 21), the total sugar consumption was 12.25 g/L. The maximum product yield in all the assay was 0.51 g/g in 22h, this value was the highest ever obtain for the bacterium in this study as well in other performed studies (table 7).

It was also detected the presence of formic acid (7.77 mM), acetic acid (17.22 mM) and 5HMF (0.004 ± 0.001 g/L) in the medium, none of these components seems to have influenced the development and production of EPS by the bacterium.

The EPS recovered from the broth at the end of the experiment was composed of fucose (40 mol %), glucose (29 mol %), galactose (26 mol %) and glucuronic acid (4 mol %), a sugar profile that was in accordance with that reported for FucoPol, despite the slightly higher fucose content and lower glucuronic acid content.

The use of acid hydrolysis to obtain a substrate based on grape pomace proved to be feasible and it was suitable to produce EPS, exhibiting very good results in terms of volumetric productivity. Although some investment is required for the hydrolysis step, the costs associated with hydrolysis may be overcome by the higher productivity obtained.

3.2.2.3. Conclusion

According to the results, using subcritical water to extract sugars and hydrolyze lignocellulosic material from grape marc to obtain simple sugars for use as a substrate for *Enterobacter* A47 was not successful, at least under the conditions tested. The concentration of sugars in the substrate was too low most likely due to the heterogeneity of the grape pomace. With the results obtained it was also concluded that in extraction with subcritical water that there was no hydrolysis, perhaps if the extraction is done at a higher temperature or raise a little the amount of powder inside of the reactor, better results can be obtained.

On the other hand, the use of an acidic hydrolysis to hydrolyze the lignocellulosic material of the grape pomace, to obtain simple sugars for use by *Enterobacter* A47 was successful, and the amount of simple sugars in the substrate was adequate for cell growth, although lower, and very good FucoPol productivity.

This study showed that *Enterobacter* A47 can use several types of sugar, which had never been tested before (diz quais), to produce FucoPol and that the bacterium does not necessitate large amounts of sugar present in the medium to be able to grow and produce EPS

Chapter 4

4. General conclusion

In this work, it was demonstrated that *Enterobacter* A47 was able to use the two-food processing wastes to produce FucoPol with a high fucose content (≈ 40 mol %), reaching productivity values never reported in previous studies. The ability of the bacterium to utilize other sugars, namely, arabinose, trehalose, rhamnose and galacturonic acid in the synthesis of EPS, was also demonstrated. Interestingly, the bacterium was further able to grow and produce FucoPol in a medium with a high salt concentration (about 50 g/L) without impacting the productivity or altering the polymer's composition. This feature had never been evaluated.

Chapter 5

5. Future work

For future work, in the case of apple pomace, although the productivity reached was already higher than in previous studies, it may eventually be further improved by optimizing substrate feeding rate or operating under different mode (e.g., pulse feed, or continuous operation). It would also be interesting to analyze the polysaccharides present in the soluble fraction of the apple pomace and evaluate their potential use and commercial value. Another relevant issue is to try to valorize the insoluble fraction obtained by centrifuging/filtering apple pomace. Maybe it can be hydrolyzed, similarly to what was tested for grape pomace, to obtain a sugar rich solution that can be then used by the culture.

In the case of grape pomace, higher temperatures can be tested for its extraction/hydrolysis with HCW to obtain a richer solution in terms of sugars. Regarding the acid hydrolysis, it may be optimized in terms of hydrolysis conditions (acid concentration, reaction time, etc.) to maximize lignocellulose hydrolysis and minimizing monosaccharides' degradation. Similarly to apple pomace, FucoPol production may be further increased by optimizing the cultivation conditions.

Chapter 6

6. References

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